Chapter II

Effects of the generation of single-stranded DNA on the maintenance of plasmid pMV158 and derivatives in *Lactococcus lactis*

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SUMMARY

The effects of the single-strand origins (SSOs) of the broad-host-range streptococcal plasmid pMV158 on (i) the conversion of its single-stranded (ss) DNA replication intermediates to double-stranded (ds) plasmid DNA and (ii) its maintenance, were analyzed. pMV158 is distinguished from most other plasmids that replicate by the rolling-circle mechanism by the presence of two single-strand origins (SSOs) of replication, palA and palU. In this paper the results obtained with Lactococcus lactis are presented; complementary studies with Bacillus subtilis are presented in chapter III. In the presence of both SSOs, no ss plasmid DNA was observed in L.lactis. The removal of either palA or palU resulted in the appearance of low amounts of ssDNA. High amounts of ssDNA were detected, however, when both SSOs were deleted. The results indicated that both SSOs were active, albeit that palU was the most effective of the two. In the presence of both SSOs the plasmid was stably maintained in L.lactis under non-selective growth conditions. Also, the derivatives containing only one of the two SSOs were maintained rather stably. In contrast, the derivative devoid of both SSOs was poorly maintained. It was concluded that, in the absence of a functional SSO, the generation of large amounts of ssDNA drastically reduces the maintenance of pMV158 in L.lactis. The results also showed that the presence of the plasmid-located mob gene, required for conjugative mobilization, was neither involved in the generation of ssDNA nor in the maintenance of pMV158.

INTRODUCTION

Many small plasmids isolated from Gram-positive bacteria are able to replicate in more than one host (Lacks et al., 1986; del Solar et al., 1987; Gruss and Ehrlich, 1989; del Solar et al., 1993b). The host-range of some of these plasmids, for instance pMV158 (Lacks et al., 1986; del Solar et al., 1993b) and pWV01 (Leenhouts et al., 1991) extends even across the Gram stain barrier. Vectors that contain the replication functions of such plasmids are potential natural shuttle vectors. However, high levels of instability, both structural and segregational, are often observed when these plasmids are used as cloning vectors, especially in non-native hosts (Ehrlich et al., 1986; Bron et al., 1988a; Bron et al., 1991b; Bron et al., 1991a; Kiewiet et al., 1993).

The observation that most of the small plasmids from Gram-positive bacteria replicate by a rolling-circle mechanism (RCM) (te Riele et al., 1986a; te Riele et al., 1986b; Gruss and Ehrlich, 1989; Novick, 1989) was a major step forward in the understanding of the observed instabilities. This replication mechanism is characterized by the production of single-stranded (ss) plasmid DNA intermediates. Efficient conversion of these replication intermediates to double-stranded (ds) plasmid DNA requires a single-strand origin (SSO, formerly called minus origin). Three different groups of SSOs have been described: the palA-, palU- and palT-types (Chang et al., 1987; Gruss et al., 1987; Boe

et al., 1989; Devine et al., 1989). All of these rely on the host-encoded RNA-polymerase for the priming of the complementary strand synthesis on the ssDNA template.

SSOs are usually only functional in their native hosts (Gruss et al., 1987; del Solar et al., 1987). Without a functional SSO, inefficient conversion of ssDNA occurs, resulting in the accumulation of circular ssDNA monomers in the cell and in reduced maintenance of several RCM plasmids in Gram-positive bacteria (Gruss et al., 1987; del Solar et al., 1987; Bron, 1990; Bron et al., 1991b).

The major aim of the present studies was to analyze the relation between the accumulation of ssDNA and the maintenance of pMV158 derivatives in L.lactis and B.subtilis. pMV158 has several properties of interest for these studies. First, this small (5.5 kb) plasmid has a broad-host-range; it replicates in several Gram-positive bacteria, such as Streptococcus agalactiae (Burdett, 1980), L.lactis and B.subtilis, and in the Gram-negative bacterium Escherichia coli (Lacks et al., 1986). Second, unlike most other RCM plasmids, pMV158 contains two SSOs, one of the palA-type and the other of the palU-type (van der Lelie et al., 1989). The latter SSO is absent from pLS1, a pMV158 derivative from which a 1.1 kb EcoRI fragment was deleted (Lacks et al., 1986; del Solar et al., 1987).

In this chapter the results obtained with L.lactis are described; those obtained with B.subtilis are presented in chapter III.

RESULTS

Construction of pMV158 derivatives.

Derivatives of pMV158, lacking either palU, palA or both SSOs, were constructed (Figure 1) to study the effects of these SSOs on ssDNA conversion and plasmid maintenance. In the nomenclature used for the constructs the letters "U" and "A" symbolize the palU and palA SSO, respectively. The presence or absence of an SSO is indicated by U^+ (or A^+) and U^- (or A^-), respectively. Unless mentioned otherwise, the derivatives were constructed in B.subtilis. Subsequently, plasmid DNA was isolated from B.subtilis transformants containing the correct construct and introduced into L.lactis strain IL1403 by electrotransformation.

pMV(U^-A^+) (=pLS1) was constructed as described by Stassi et al. (1981) by in vitro deletion of the 1.14-kb EcoRI fragment from pMV158. In addition to palU it lacks the promoter and the first 190 codons of the mob gene (Priebe and Lacks, 1989; van der Lelie et al., 1990). In pMV(U^+A^-) the 1.55-kb AvaI fragment, that besides palA also contains the major 3'-terminal part of the mob gene (458 codons), was deleted. pMV(U^-A^-) was constructed from pMV(U^+A^-) by deleting the 0.66 kb-EcoRI/AvaI fragment. The 5' overhanging ends of the largest EcoRI/AvaI fragment were filled-in using Klenow DNA polymerase after which this fragment was circularized by ligation. As expected, a unique EcoRI site was formed by the joining of the filled-in EcoRI and AvaI sites. pMV(U^-A^-) lacks both SSOs as well as the complete mob gene. pMV(mob^*), encoding an inactive Mob protein, was constructed by filling in the unique HindIII site of pMV158, resulting in the formation of a unique NheI site and a frameshift at the 228^{th} codon of the mob gene. To replace the mob gene by the Cm^R gene from Bacillus pumilus, pMV158 was digested with BfrI

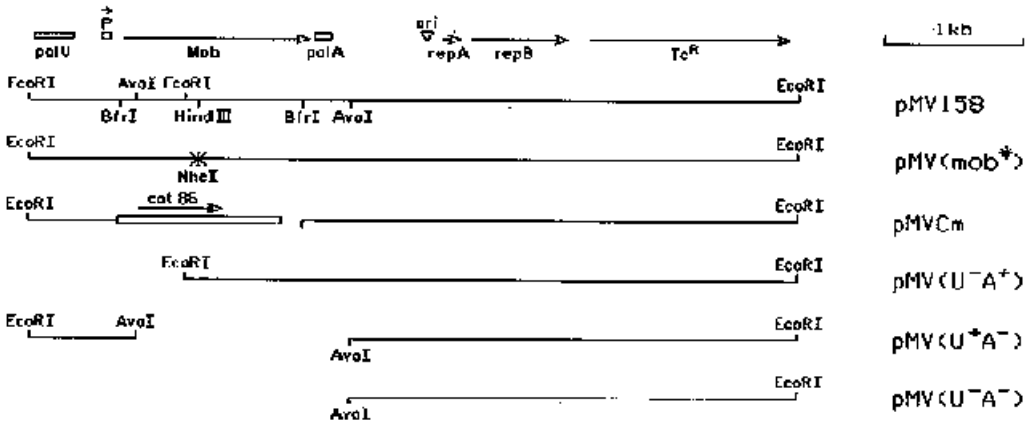

Figure 1. Linear restriction maps of pMV158 and derivatives.
The positions of palU, the mob gene (Mob) and its promoter (p), palA, the double-strand origin (ori), the replication region (repA and repB), and the tetracycline resistance gene (Tet) are indicated above the map of pMV158. Only restriction sites relevant for constructions are indicated.

and pGKV110, which contains this Cm^R gene (van der Vossen et al., 1985) with BstYI. The 4.1 kb BfrI fragment of pMV158 and the 1.3 kb BstYI pGKV110 fragment, containing the Cm^R gene, were purified and the ends were filled-in. A ligation mixture of the two fragments was used for transformation. As checked by restriction analysis of chloramphenicol resistant transformants, the desired plasmid, pMVCm, was obtained.

Effect of the minus origins and the mob gene on plasmid copynumbers

The copy numbers per chromosome equivalent of the different constructs were determined from the ratio between radio-active labelled plasmid and chromosomal DNA. The averages of three independent experiments are listed in Table 1. The differences in copy numbers are not significant which is also a consequence of the large variation between results from independent experiments. If any effect of SSO removal existed, this tended to be in the direction of slightly increased copy numbers.

Effect of the single-strand origins and the mob gene region on the accumulation of ss plasmid DNA

The effects of palU and/or palA, and of an intact mob gene on the efficiency of conversion of ss to ds plasmid DNA were examined. Total DNA was isolated from cells harbouring pMV158 or its derivatives. Each DNA preparation was divided into two portions, one of which was treated with S1-endonuclease to digest ssDNA if present, and the other was left untreated. The mixtures were subjected to agarose gel electrophoresis and, after denaturation and neutralization, to Southern hybridization. The results are shown in Fig. 2. Deletion of both SSOs together with the mob gene [pMV(U^-A^-)] resulted in a high level of accumulation of ssDNA. With pMV158 and pMV(mob^*), which contain both SSOs, no ssDNA could be detected. With pMV(U-A+) ssDNA was observed but the amount was considerably less than in cells carrying

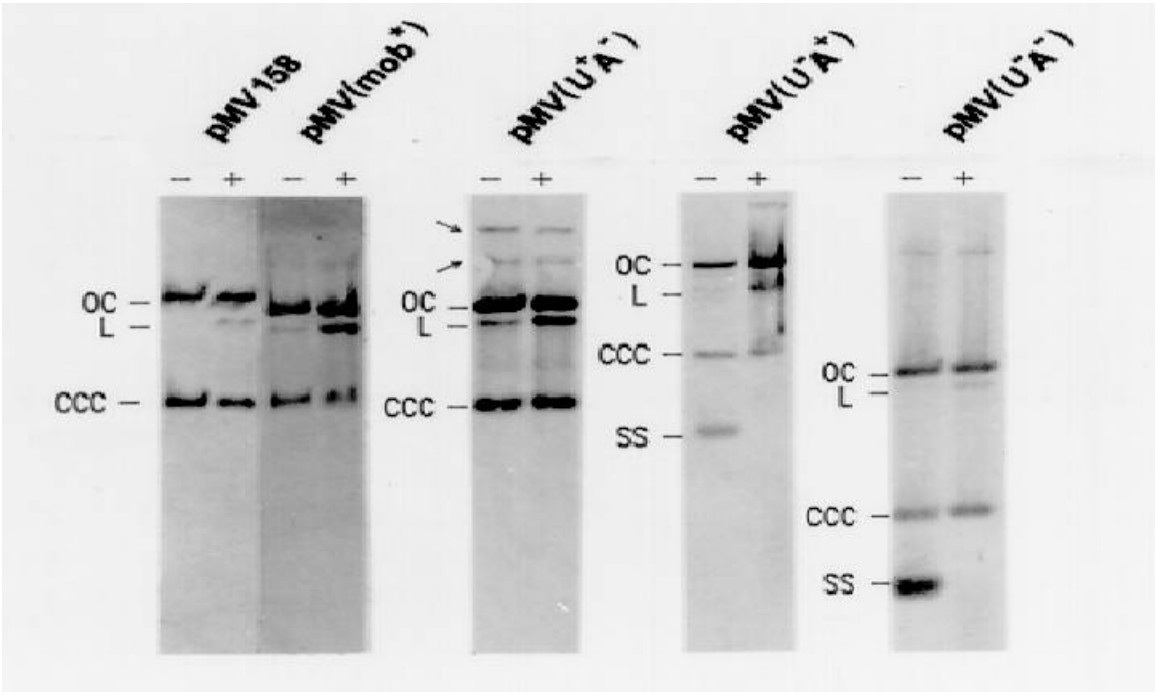

Figure 2. Southern hybridizations of total DNA preparations of L.lactis IL1403 harbouring various plasmids.
The plasmid preparations were incubated without (-) or with (+) endonuclease S1. The blots were hybridized with randomly labelled pMV158 DNA. The positions of single-stranded DNA (SS), covalently closed circular DNA (CCC), linear DNA (L) and open circular DNA (OC) are indicated. Arrows indicate

pMV(U^-A^-). When only palU was present, as in pMV(U^+A^-), no ssDNA was observed under the standard conditions. However, some ssDNA could be detected with pMV(U^+A^-) when the gels were overloaded (results not shown). With pMV(U^+A^-) some plasmid DNA from cells harbouring pMV(U^+A^-) migrated to positions corresponding to DNA forms of increased molecular weight (indicated with arrows) . The observation that the absence of both SSOs resulted in far higher levels of ssDNA than the deletion of each of these SSOs separately, indicates that both SSOs are functional in the conversion of ss- to dsDNA in L.lactis. Furthermore, the results showed that palU was the most efficient of the two SSOs (less ssDNA in the presence of palU as compared to palA).
Inactivation of the mob gene, as in pMV(mob*), did not interfere with the efficient conversion of ssDNA [Fig. 2, compare pMV158 and pMV(mob*)], nor did the replacement of the mob gene by the Cm^R gene (pMVCm, results not shown). These results indicate that neither the presence of functional Mob protein, nor the mob gene region, were required for the initiation of lagging strand synthesis in pMV158.

Effects of the mob gene region on plasmid maintenance

Previous studies showed that deletion of an internal DNA fragment from the mob gene of plasmid pUB110 resulted in segregational instability of large pUB110 derivatives in B.subtilis, although no effect on ssDNA accumulation was observed (Bron et al., 1988b). From those studies it was not clear whether the decreased stability was due to inactivation of the Mob protein, or to the deletion of structural elements located within the mob gene. To

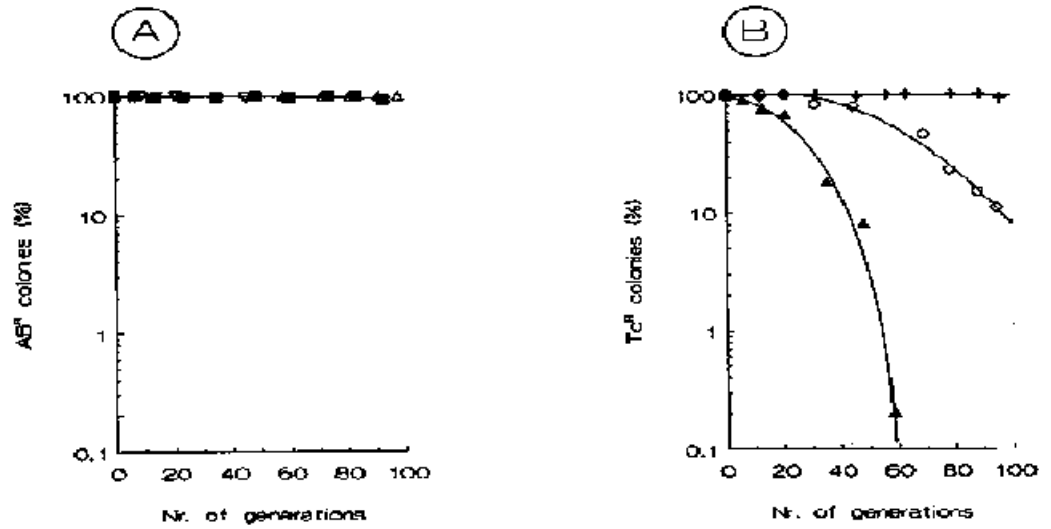

Figure 3. Kinetics of plasmid loss in L.lactis IL1403.
(A): Effects of inactivation or replacement of the mob gene on plasmid maintenance. # : pMV158; ª : pMV(mob*); « : pMVCm. (B): Effects of deletion of SSOs on plasmid maintenance. + : pMV(U^-A^+); O : pMV(U^+A^-); > : pMV(U^-A^-).

test whether either of these possibilities could play a role in the system described here, we measured the maintenance of pMV158, pMV(mob*) and pMVCm (Fig. 3A). Within the same experiment also the maintenance of the constructs lacking one or both SSOs were tested; for clarity these results, described below, are presented in Fig. 3B. As shown in Fig. 3A, pMV158 was maintained stably, and neither the inactivation of the mob gene [pMV(mob*)], nor the replacement of this gene by the Cm^R-gene (pMVCm), reduced the level of plasmid maintenance within the test period (100 generations of growth in non-selective medium). However, plasmid-free cells were observed at a low frequency (< 5%) in all these cultures, also during early stages of the experiments.

Effects of the single-strand origins on plasmid maintenance

The effects of the SSOs on plasmid maintenance were tested with pMV(U^-A^+), pMV(U^+A^-) and pMV(U^-A^-). The results (Fig. 3B) showed that deletion of both SSOs [pMV(U^-A^-)], resulted in a severe decrease of stability: less than 1% of the cells contained the plasmid after about 50 generations of growth. Therefore, with pMV(U^-A^-), the accumulation of high amounts of ssDNA paralleled a high decrease in plasmid maintenance. Also, the maximal growth rate of L.lactis cells harbouring pMV(U^-A^-) was clearly lower than that of cells harbouring one of the other derivatives (results not shown).
pMV(U^+A^-) and pMV(U^-A^+) were maintained considerably better than pMV(U^-A^-). After 100 generations of growth approximately 20% [pMV(U^+A^-)] and 90% [pMV(U^-A^+)] of the cells were tetracycline resistant. Apparently, accumulation of large amounts of ssDNA, as with pMV(U^-A^-), had a drastic effect on maintenance. However, although the plasmid tended to be lost when detectable amounts of ssDNA were observed, no strict

correlation between the amount of ssDNA and maintenance existed. This last conclusion is based on the observation that although pMV(U^-A^+) accumulated more ssDNA than pMV(U^+A^-), the former was the most stable of the two plasmids.

DISCUSSION

The major conclusions from the results presented in this paper are that, in L.lactis, the two SSOs of the broad-host-range plasmid pMV158 are functional in the conversion of ssDNA replication intermediates to duplex plasmid DNA and that, in the absence of both SSOs, inefficient ssDNA conversion leads to a severe decrease in plasmid maintenance. These effects of the pMV158 SSOs are discussed below.

Effects of the SSOs of pMV158 on ssDNA conversion.

The results showed that, although both SSOs of pMV158 are active in the conversion of ss to ds plasmid DNA in L.lactis, palU is the most efficient of the two. SSOs, especially those of the palA type, were described to be mainly functional in their native hosts (Gruss et al., 1987; del Solar et al., 1987). The results presented here showed that the functionality of palA and palU of pMV158 is not limited to their native host. In addition to being functional in S.pneumoniae (del Solar et al., 1987; del Solar et al., 1993a), palA is also clearly functional in L.lactis. The host-range for palU activity is even wider. In addition to its known activity in S.aureus and B.subtilis (Bron et al., 1988b; Boe et al., 1989; Meijer et al., 1995), palU is clearly also functional in L.lactis. The reason for the activity of these SSOs in at least a number of Gram-positive bacteria is not known. Since these SSOs depend on the host-encoded RNA polymerase for the initiation for lagging strand synthesis (Gruss et al., 1987; Boe et al., 1989), an obvious explanation their activity in several hosts is that this priming reaction is not very host specific. Sofar, we have not tested this possibility.

Effects of the SSOs of pMV158 on plasmid maintenance

A high degree of plasmid maintenance was observed when no ssDNA was detected, as was the case with cells harbouring either pMV158, pMV(mob*) or pMVCm. Small numbers of plasmid-free cells (<5%) were, however, present in these cultures even during the early stages of the experiments. These cells did not outgrow the plasmid-containing cells, indicating that no substantial growth differences existed between plasmid-free cells and cells harbouring pMV158, pMV(mob*) or pMVCm. This makes it unlikely that the presence of the Mob protein or part of the mob gene affected the maintenance of pMV158 in L.lactis by affecting cellular growth rates.

The observation that pMV(U^-A^-), lacking both SSOs and generating large amounts of ssDNA, was unstably maintained, is in agreement with previous reports indicating that SSOs can play a significant role in the stable maintenance of RCM plasmids in Gram-positive bacteria (del Solar et al., 1987; del Solar et al., 1993a; Bron et al., 1988a; Bron et al., 1988b; Bron et al., 1991b; Viret and Alonso, 1988; Bron, 1990). At least two explanations can be entertained for these observations. The first is based on reduced plasmid copy numbers. With certain plasmids in S.aureus (Gruss et al., 1987), and with pMV158 in S.pneumoniae (del Solar et al., 1987), the copy number of ds plasmid forms is reduced in the absence of a functional SSO. Conceivably, in these cases, the ssDNA intermediates are not efficiently converted to ds plasmid DNA so that the plasmid copy number drops. In the present work no decrease in plasmid copy

number was observed with the deletion derivatives used here. This suggests that the reduced plasmid maintenance in this case is not due to reduced plasmid copy numbers. It should be noted, however, that determinations of the cell average copy numbers do not give information about possible variations in the distribution of plasmid copies per cell. We can therefore not exclude the possibility that deviations from the average copy numbers are higher when the SSO is absent and that an increased frequency of cells with a lower than average number of plasmid copies accounts for high plasmid loss rates. A second possible explanation for the reduced plasmid maintenance in the absence of SSOs is that high amounts of ssDNA are deleterious to the cell and result in reduced growth rates (Bron et al., 1988a; Viret and Alonso, 1988). We favour this explanation for the present results. It is likely that the accumulation of large amounts of ssDNA caused the reduction in growth rate observed with cells harbouring pMV(U^-A^-). These cells will be outcompeted by plasmid-free cells which have normal growth rates. The biphasic nature of the kinetics of loss of pMV(U^-A^-) (Fig. 3B) is indicative for outgrowth by plasmid-free cells (Boe et al., 1987).

When low amounts of ssDNA were accumulated, no correlation between the amounts of ssDNA and plasmid maintenance was observed: although pMV(U^+A^-) accumulated less ssDNA than pMV(U^-A^+), it was the most unstable of the two plasmids. This indicates that factors other than the formation of ssDNA cause instability of pMV(U^+A^-). One possible factor is the absence of the 3' part of the mob gene from pMV(U^+A^-). Although inactivation [pMV(mob^*)], or replacement of the mob gene by a Cm^R-gene (pMVCm) were shown not to affect plasmid maintenance, the results do not entirely rule out the possibility that the observed instability of pMV(U^+A^-) is caused by additive effects of the joint deletion of palA and the 3' part of the mob gene. We favour another explanation, however, which is based on the observation that low amounts of DNA of increased molecular weight were generated by pMV(U^+A^-). Studies by others indicated that the formation of multimeric plasmid DNA in E.coli (Summers and Sherratt, 1984; Kusano et al., 1989), and linear high-molecular-weight DNA in B.subtilis (Gruss and Ehrlich, 1988) resulted in increased plasmid instability. In view of these observations it is tempting to suppose that the high-molecular-weight plasmid forms observed with pMV(U^+A^-) also affected the plasmid stability in the present system.

In conclusion, our results showed that the accumulation of large amounts of ss plasmid DNA, caused by absence of functional SSOs, is an important factor in the maintenance of pMV158 in L.lactis. Since this corroborates earlier findings with other RCM plasmids in Gram-positive bacteria, it seems that this form of plasmid instability is generally associated with plasmids using the rolling-circle mode of replication when functional SSOs are lacking. However, the results also showed that in addition to the generation of ssDNA, also other factors can play a role in the instability of RCM plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids and media . Bacterial strains and plasmids used are listed in Table I. TY medium, used for culturing E.coli and B.subtilis, contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). L.lactis was cultured in M17 broth (Terzaghi and Sandine, 1975) supplemented with 0.5% glucose (GM17). Plates contained, in addition, 2% agar. Tetracycline was added to a final concentration of 4 µg/ml for L.lactis and 10 µg/ml for B.subtilis and

Table 1. Plasmids and strains

Plasmid	size[a]	Relevant properties	Copy number[b]	Reference
pMV158	5.5	palA, palU, mob-gene, Tc^R	5.0 ± 2.8	Burdett, 1980
pMV(U^-A^+)	4.4	palA, Tc^R	9.7 ± 4.1	Lacks et al., 1986
pMV(U^+A^-)	3.9	palU, Tc^R	8,4 ± 4.9	This study
pMV(mob^*)	5.5	palA, palU, inactive Mob, Tc^R	3.7 ± 2.6	This study
pMV(U^-A^-)	3.2	Tc^R	7.4 ± 3.9	This study
pMVCm	5.4	palA, palU,△ mob-gene, Tc^R, Cm^R	7.8 ± 2.9	this study
pGKV110	4.7	Cm^R, pWV01 derivative	nd[c]	van der Vossen et al., 1985

Bacterial strain	Genotype	Reference
L.lactis		
IL1403	Plasmid-free strain	Chopin et al., 1984
B.subtilis		
PSL1	leuA8 arg15 thrA recA4 r^-_M m^-_M	Ostroff and Pène, 1983
E.coli		
JM83	F- ara △(lac-proAB) rpsL (Str^r) (Φ80d△(lacZ)M15)	Vieira and Messing, 1982

a: size in kb.
b: copy number per chromosome equivalent; average of three experiments
c: not determined

E.coli. Chloramphenicol was added to a final concentration of 10 µg/ml.

DNA techniques. All DNA manipulations were carried out according to Sambrook et al. (1989). Restriction enzymes were used as indicated by the supplier. Plasmid DNA was isolated from E.coli as described (Sambrook et al., 1989) and, with minor modifications of the same procedure, from L.lactis (van der Lelie and Venema, 1987). Total DNA lysates were prepared as described before (Bron et al., 1988b). DNA fragments were isolated from agarose gels using DEAE NA-52 membranes (Schleicher and Schuell, Dassel, Germany). GeneScreen Plus filters (Dupont NEN, Boston, Ma.) were used for Southern transfers of DNA. For the production of the hybridization probes random-primed DNA was labelled with digoxigenin-dUTP using the nonradioactive DNA labelling and detection kit (Boehringer GmbH, Mannheim, Germany). The probes were subsequently denatured for 10 min at 100°C. The hybridization and staining steps were carried out according to the manufacturer's instructions.

To quantify the amounts of ssDNA, total DNA lysates were separated by agarose gel electrophoresis. After Southern blotting and hybridization with plasmid probes, the relative density of each plasmid band wasdetermined using the LKB 2400 Gelscan XL (Pharmacia, Uppsala, Sweden). The relative amounts of ssDNA are given as percentage of the total absorption in all plasmid bands.

Transformation of L.lactis, B.subtilis and E.coli. L.lactis was transformed by electrotransformation as described before (van der Lelie et al., 1989). Competent cells and protoplasts of B.subtilis were prepared and transformed as described (Bron, 1990). E.coli was transformed as described before (Sambrook et al., 1989).

Copy number determination. Copy numbers of pMV158 and derivatives were determined using [methyl-^{3}H]thymidine as described (Bron, 1990). To visualize the plasmid bands, 5 µl of the radioactive samples were mixed with 1 µg of non-labelled plasmid DNA before loading onto 0.8% agarose-gel. Copy numbers were calculated per chromosome equivalent. The genome size of L.lactis IL1403 was taken to be 2.42×10^6 bp (Le Bourgeois et al., 1992).

Assay of plasmid maintenance. Plasmid maintenance was assayed by culturing the cells in the absence of selective antibiotics for about 100 generations as described (Bron, 1990). Fifty antibiotic-resistant and fifty antibiotic-sensitive colonies were checked for the presence or absence of the plasmid. As expected, in all antibiotic-resistant colonies plasmid DNA was detected, whereas all the antibiotic-sensitive colonies were plasmid-free.

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