Replication and maintenance of plasmids in Bacillus subtilis

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Chapter IV

Alternative, RecA-mediated pathway for the initiation of lagging strand synthesis of the broad-host-range rolling-circle plasmid pMV158 in Bacillus subtilis

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SUMMARY

Pathways for the initiation of lagging strand synthesis of the broad-host-range rolling-circle plasmid pMV158 in Bacillus subtilis were analyzed. The major pathway for the conversion of the single-stranded (ss) DNA replication intermediates to double-stranded plasmid DNA involves RNA-polymerase-dependent priming at the initiation signals for ssDNA conversion, designated single strand origins (SSOs). Although deletion of the plasmid regions containing the pMV158 SSOs resulted in the accumulation of ss plasmid DNA in the cell and plasmid instability, these plasmids were still able to replicate. This indicates that an alternative pathway for the initiation of lagging strand synthesis of pMV158 does exist. Our results show that pMV158 derivatives lacking an SSO accumulate substantially more ssDNA in RecA-deficient than in RecA-proficient B.subtilis strains. In addition, a plasmid region encoding a small RNA transcript which is complementary to the ssDNA plasmid intermediates, drastically reduced ssDNA accumulation. These results indicate that RecA and the complementary RNA are involved in the alternative ssDNA conversion route. A model is proposed for this pathway, in which small RNA transcripts anneal to complementary ssDNA molecules in a RecA-stimulated way, resulting in the formation of an initiation complex for lagging strand synthesis.

INTRODUCTION

Most cloning vectors used today for Bacillus subtilis are based on small plasmids originally isolated from other Gram-positive bacteria, like pUB110, pE194, pT181 and pC194 from Staphylococcus aureus; and pMV158 from Streptococcus agalactiae. These plasmids replicate via the rolling-circle mechanism (RCM) similar to that of the Escherichia coli phages ΦX174 (Novick, 1989; Gruss and Ehrlich, 1989; Jannière et al., 1993). Characteristically, this type of replication involves: (i) the introduction of a strand- and site-specific nick by the plasmid-encoded replication protein (Rep); (ii) the synthesis of the leading strand through extension of the 3' end at the nick site and displacement of the original leading strand; and (iii) the conversion of the displaced leading single strand into a duplex plasmid DNA molecule. Initiation of conversion of the displaced ssDNA strand normally occurs at a specific, non-coding, plasmid sequence which has the potential to form secondary structures. These sites are called single strand origins (SSOs). Most known SSOs can be classified in three families: the palA, palU and palT family (Gruss et al., 1987; Boe et al., 1989; Bron, 1990; Meijer et al., 1995c). Since the activity of most known SSOs depends on the host-encoded RNA-polymerase (RNAP) (Boe et al., 1989; Birch and Khan, 1992; Seery and Devine, 1993; Dempsey et al., 1995), it is believed that RNAP synthesizes a primer on the SSO which is, subsequently, used for lagging strand synthesis. This idea has been confirmed for pT181, pE194, pSN2 and pUB110 by in vitro replication studies (Birch and Khan, 1992; Dempsey et al.,

1995). With the exception of palU, SSOs are normally only functional in their native or related hosts (Gruss et al., 1987; del Solar et al., 1987; Kramer et al., 1995; Meijer et al., 1995a; Meijer et al., 1995b). Most plasmids from S.aureus contain a palA-type SSO which is not or only poorly functional in B.subtilis (del Solar et al., 1987; Kramer et al., 1995; Meijer et al., 1995b). Consequently, ssDNA conversion with these plasmids in B.subtilis occurs inefficiently and results in the accumulation of ssDNA molecules. Although exceptions are known (del Solar et al., 1993a), ssDNA accumulation generally leads to plasmid instability (Ehrlich et al., 1986; Gruss et al., 1987; del Solar et al., 1987; Bron et al., 1991a).

A general observation in this and other laboratories is that even in the absence of a functional SSO, RCM plasmids are still able to replicate. This implies that ssDNA can be converted to duplex DNA via an alternative, SSO-independent, pathway. The aim of the present work was to analyze the nature of this pathway for the conversion of pMV158 ssDNA to duplex DNA. In particular, we were interested in the role of RecA, which is the key enzyme in DNA recombination and repair processes, in the presumed alternative ssDNA conversion pathway. The rational for this approach was that our previous preliminary results indicated that derivatives of pMV158 lacking a functional SSO accumulated more ssDNA in a RecA-deficient compared to an isogenic RecA-proficient B.subtilis strain (Meijer et al., 1995b). The present results indicate that RecA is indeed involved in an alternative ssDNA conversion pathway in which a small transcript RNA that is complementary to the leading strand plays an important role.

RESULTS

ssDNA accumulation with pMV158 derivatives containing or lacking SSOs in recA4 and wild-type recA backgrounds

pMV158 is exceptional among RCM plasmids in the sense that it contains two SSOs, one of the palA and one of the palU type. Only palU is efficiently used in B.subtilis (del Solar et al., 1987; Boe et al., 1989; Meijer et al., 1995b). We have shown previously that deletion derivatives of pMV158 lacking both SSOs accumulated only moderate amounts of ssDNA in B.subtilis 8G5 cells, indicating that ssDNA conversion still occurred rather efficiently (Meijer et al., 1995b). However, these plasmids accumulated large amounts of ssDNA in isogenic recA4 mutants (Meijer et al., 1995b), suggesting that RecA might be involved in the conversion of ssDNA to duplex plasmid DNA. In the present work we wanted to obtain support for this idea and to analyze the nature of the alternative, SSO-independent, pathway of ssDNA conversion of pMV158 derivatives. For this purpose we used, in addition to the parental plasmid pMV158, plasmid pMV(U^-A^-)Em, which is a spontaneous erythromycin-resistant high-copy number derivative (about 100 copies per chromosome equivalent) of pMV158 (Fig. 1). pLW5, pLW6 and pLW7, also shown in Fig. 1, will be discussed in one of the following sections. pMV(U^-A^-)Em lacks all DNA sequences that might exert SSO activity (Meijer et al., 1995b). An advantage of this plasmid and its derivatives is that, due to the high copy number, they can establish themselves even when ssDNA conversion occurs very inefficiently. Moreover, the high copy number of pMV(U^-A^-)Em facilitated the determination of differences in ssDNA accumulation.

We have used an isogenic set of PSL1 derivatives. Like 8G5, strain PSL1 is

a derivative of B.subtilis 168 which seems to be low in DNase activity (Ostroff and Pène, 1983) which may be advantageous for accurately assessing the presence of ss plasmid DNA. PSL1 contains the recA4 allele, which specifies a non-functional RecA4 product (de Vos et al., 1983; Ceglowski et al., 1990). In strain PSL161, the wild-type recA gene is present on plasmid pBT61 in about five copies per

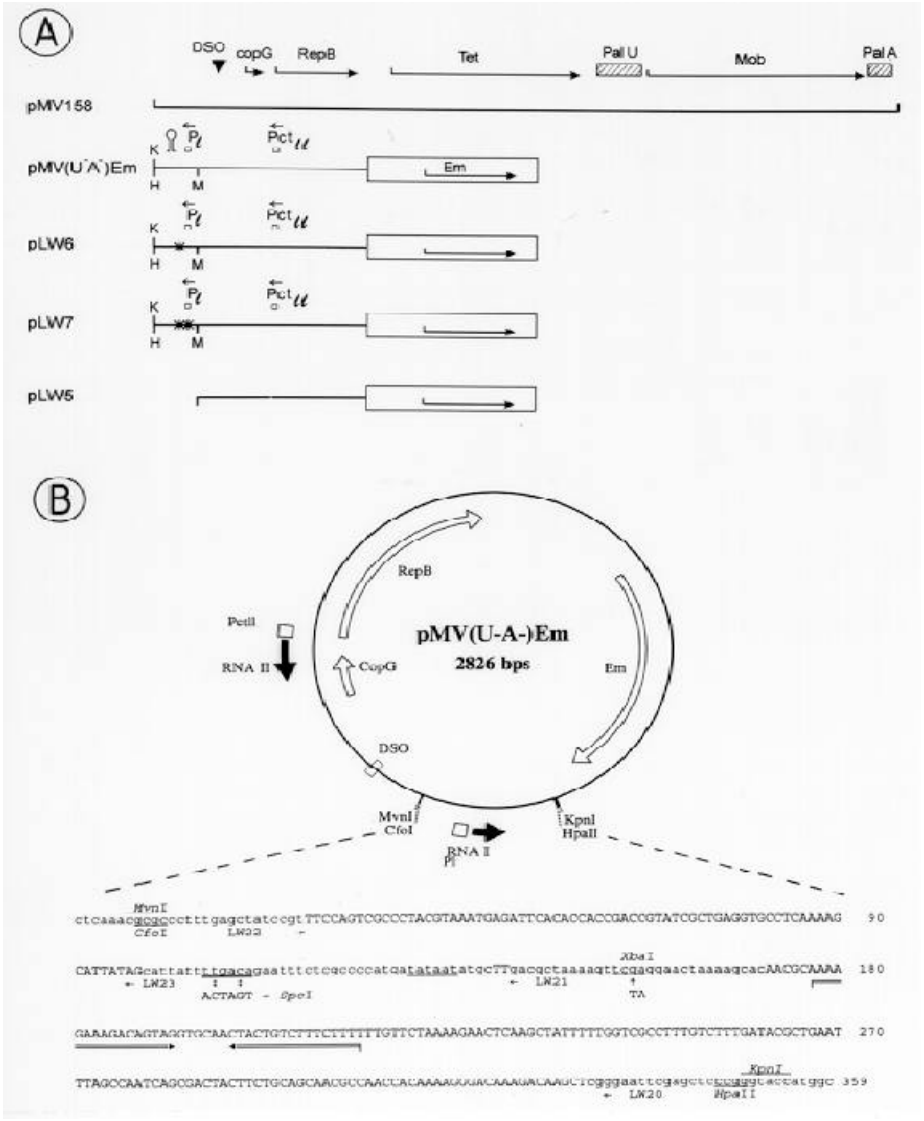

Figure 1. Physical maps of pMV158 and the derivatives pMV(U-A-)Em, pLW5, pLW6, and pLW7.

(A) Linear maps of pMV158, pMV(U-A-)Em, pLW5, pLW6, and pLW7. The positions of promoter P_1, promoter$_{ctII}$ P (PctII), the double-strand origin (DSO), the replication region (CopG and RepB), the tetracycline-resistance gene (Tet), palU, the mob gene (Mob) and palA are indicated above the map of pMV158. Regions originating from pE194 are indicated as open bars; the position of the erythromycin resistance gene is shown (Em). DNA alterations in pLW6 and pLW7 are indicated with asterisks. Only relevant restriction sites are indicated: K, KpnI; H, HpaII; M, MvnI. The region containing secondary structure is indicated with a stem loop.

(B) pMV(U-A-)Em. (Top): Circular map of pMV(U-A-)Em. Relevant properties are indicated using the same nomenclature as in (A). Transcripts RNAI and RNAII are indicated with filled arrows. HpaII, KpnI, CfoI and MvnI mark the positions of unique restriction sites. (Bottom): DNA sequence of the 0.36 kb HpaII/MvnI region of pMV(U-A-)Em. The following properties are indicated: relevant restriction sites are under- or overlined; the -35 and -10 sequences of P_1 are double underlined. The alterations in the sequences used to create a XbaI site (in plasmid pLW6) and a SpeI site (in plasmid pLW7) are indicated. The putative transcriptional terminator is indicated with double-lined arrows. Primer sequences used for the construction of pLW6 and pLW7 are shown in lowercase letters.

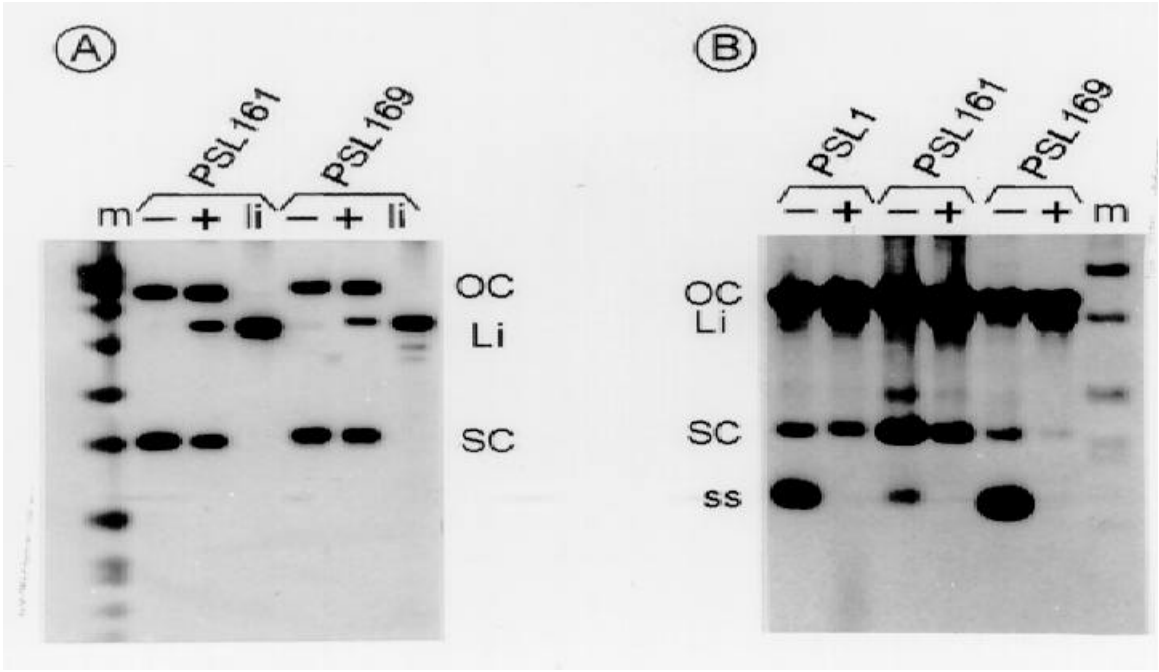

Figure 2. Southern hybridizations of total DNA preparations from logarithmically growing B.subtilis cells harbouring pMV158 (A) or pMV(UA$^-$)Em (B).
Extracts were prepared from strains PSL1 (non functional RecA), PSL161 (functional RecA), or PSL169 (non functional RecA) as indicated. Samples were incubated without (-) or with (+) S1-endonuclease or were linearized (li). The blots were hybridized with pMV(U$^-$A$^-$)Em DNA. EcoRI-digested SPP1 phage DNA was used as molecular weight reference marker (lane indicated with "m"). The positions of the various plasmid DNA forms are indicated: ssDNA (ss), supercoiled (SC), linear

chromosome equivalent. In strain PSL169 the recA gene on plasmid pBT69K was rendered defective by the insertion of a kanamycin resistance gene. pBT61 and pBT69K are both based on pUB110cop1, a low copy number derivative of pUB110 (Leonhardt, 1990).

To test whether RecA contributes to the efficiency of ssDNA conversion when the functional palU-type SSO is present on the plasmid, accumulation of ss pMV158 DNA was studied in exponentially growing cells of the B.subtilis strains PSL169 (recA4) and PSL161 (isogenic wild-type recA). For this purpose, total DNA extracts were prepared from exponentially growing cells containing pMV158. Samples of the extracts were either left untreated or treated with S1-nuclease to digest ssDNA, or with HindIII to linearize the plasmid. Next, the samples were separated by agarose gel electrophoresis and subjected to Southern hybridization. The results are shown in Fig. 2A. Under these conditions no ssDNA was detected in either strain tested. Trace amounts of ssDNA could only be detected after prolonged exposure times (results not shown). These results confirm that ssDNA conversion occurs efficiently with plasmids containing palU and demonstrate that the efficiency of this process is not detectably affected by presence or absence of the host-encoded RecA protein. Similar experiments were performed with plasmid pMV(U$^-$A$^-$)Em, which lacks a functional SSO. The results of Southern hybridizations are shown in Fig. 2B. In contrast to the results obtained with pMV158, ssDNA was clearly detectable with pMV(U$^-$A$^-$)Em and the amount of accumulated ssDNA was

drastically increased in the absence of functional RecA protein. Apparently, functional RecA protein stimulates the conversion of ssDNA to duplex DNA of pMV158 derivatives lacking a functional SSO. However, even in the RecA-deficient strains, pMV(U^-A^-)Em is still able to establish itself.

The P_1 promoter region is required for the RecA-mediated pathway of ssDNA conversion of pMV158 derivatives

One conceivable role of RecA in the SSO-independent initiation of lagging strand synthesis of pMV158 derivatives is that it stimulates the formation of a priming complex through the annealing of complementary polynucleotides to the displaced leading strand. In an attempt to obtain support for this idea we tried to identify polynucleotides complementary to the displaced ss leading strand and reasoned that plasmid-specified transcripts are potential candidates for such a function. The mRNAs transcribed from the copG/repB or Em^R genes can not fulfil this function, since these have the same polarity as the displaced ssDNA strand (Lacks et al., 1986; Puyet et al., 1988). However, two promoter-like sequences are present on pMV158 (Lacks et al., 1986) which, if active, could drive the synthesis of RNA molecules of the opposite polarity. These RNAs would thus be complementary to the ss plasmid DNA. The respective promoter sequences are indicated as P_1 and P_{ctII} in Figs. 1A and 1B. Activity of P_{ctII} has already been demonstrated (del Solar and Espinosa, 1992). Transcripts initiated from this promoter, which are antisense RNAs of the cop-rep mRNA, down regulate the plasmid copy number (del Solar and Espinosa, 1992). Demonstration of P_1 promoter activity is described in the following section.

We wanted to test whether these small putative complementary RNAs (RNAI and RNAII; Fig. 1B) might be involved in

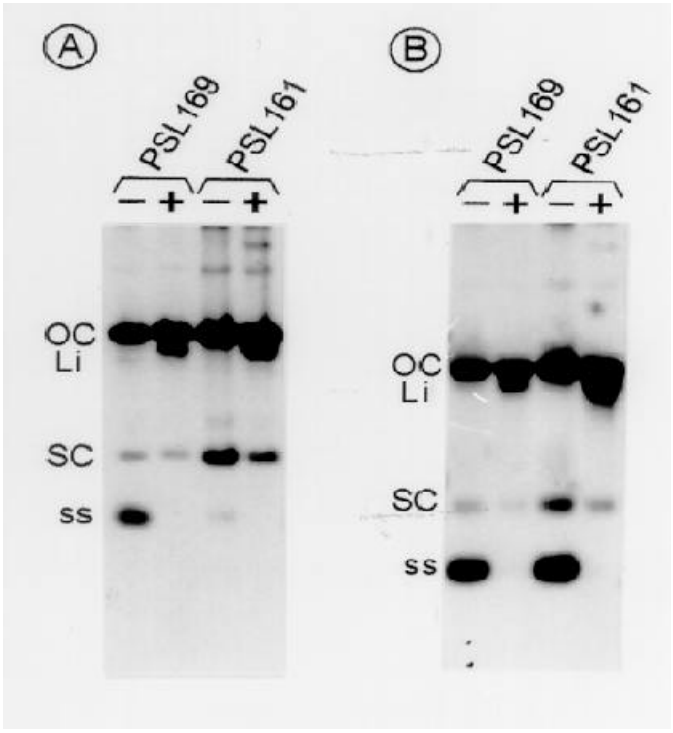

Figure 3. Effects of the presence or absence of the P_1 region on ssDNA accumulation.
Southern hybridizations of total DNA preparations from logarithmically growing B.subtilis cells harbouring pMV(U^-A^-)Em (A) or pLW5 (B) are shown. pLW5 is pMV(U^-A^-)Em lacking the 0.36 kb CfoI/HpaII fragment containing the P_1 region. Strains PSL169 (non functional RecA) and PSL161 (functional RecA) were used as hosts. Samples were incubated without (-) or with (+) S1-endonuclease. The blots were hybridized with pMV(U^-A^-)Em DNA. The positions of the various plasmid

the RecA-dependent pathway of pMV158 lagging strand synthesis. Manipulation of the region specifying RNAII would interfere with the expression of the plasmid replication initiator protein, RepB, and introduce an undesirable variable in our assays for ss plasmid DNA. Therefore, we focused on the pMV158 region specifying the putative RNAI since, unlike the RNAII region, this region is not involved in the regulation of plasmid replication. pLW5 was chosen for these studies. This plasmid is a deletion derivative of pMV(U^-A^-)Em lacking the region containing promoter P_1 and, as a consequence, the putative RNAI will not be synthesized. Total DNA extracts of logarithmically growing B.subtilis PSL161 and PSL169 cells containing pLW5 were prepared and were divided into two portions, one of which was treated with S1-nuclease and the other was left untreated. After agarose gel electrophoresis, the DNAs were analyzed by Southern hybridizations. The results (Fig. 3B) show that with pLW5 large amounts of ssDNA were present, irrespective of the presence or absence of RecA in the host cell. This contrasts sharply with the results obtained with pMV(U^-A^-)Em (Figs. 2B and 3A) which contains the P_1 promoter region. As already shown in the foregoing section, with the latter plasmid large amounts of ssDNA accumulated only in the RecA-deficient host. These results clearly demonstrate that both the RecA-proficiency of the host and the presence of the DNA region specifying the putative RNAI on plasmid pMV158 are required for the SSO-independent pathway of lagging strand synthesis.

The P_1 promoter is temporally regulated and its activity is required in the RecA-mediated pathway of lagging strand synthesis

Two explanations are possible for the accumulation of ssDNA observed with pLW5, even in a RecA-proficient background. The first is that the DNA sequences removed in this plasmid specify a component (probably RNAI) required for the alternative pathway of lagging strand synthesis, and the second that the rearrangement in the plasmid (the loss of a particular DNA sequence) rather than the lack of production of RNAI causes the high-level accumulation of pLW5 ssDNA. To discriminate between these possibilities, we tested ssDNA accumulation with a pMV(U^-A^-)Em derivative in which the P_1 promoter was inactivated. For this purpose, plasmid pLW7 was constructed in which the -35 sequence of P_1 was replaced by a hexamer sequence which constitutes an SpeI site. Details of the construction of pLW7 are described in the legends to Fig. 1B. First, we analyzed whether the altered promoter, P_1*, resulted in a decrease of promoter activity. For this purpose, transcriptional lacZ fusions were constructed with the original and the P_1* promoter (Fig. 4A). To avoid possible side effects caused by altered plasmid copy numbers, the lacZ fusions were integrated via double cross-over events in the amyE locus of the B.subtilis chromosome. ß-Galactosidase activities of the P_1-lacZ fusion (strain 8GK6), the P_1*-LacZ fusion (strain 8GK7), and the integrated promoter screening vector pBTK2 (strain 8GK2, negative control), were measured as a function of growth time of the cultures (Fig. 4B). As expected, no ß-galactosidase activity was measured with the 8GK2 control (maximum levels: 2 Miller units). From the results obtained with 8GK6 and 8GK7 three conclusions can be drawn: (i) promoter P_1 has a high activity in B.subtilis, which makes it likely that in its natural

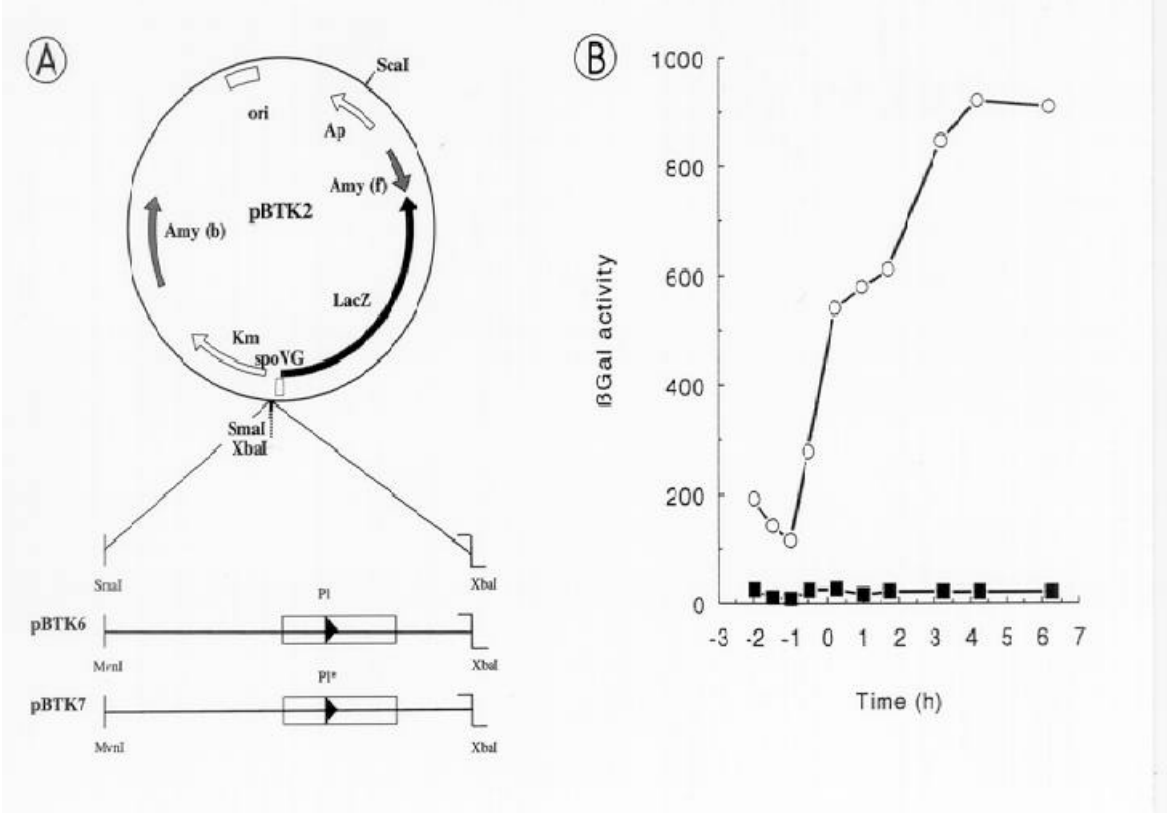

Figure 4. Growth- stage dependent expression of the P_1-lacZ and the P_1*-lacZ transcriptional fusions in B.subtilis strains 8GK6 and 8GK7.
(A) B.subtilis integrati on vector pBTK2 and derivatives. pBTK2 is derived from pE1 (Haijema et al., 1995). The following properties are indicated: ori, origin of pBR322; amy-back [Amy (b)] and amy-front [Amy (f)] refer to DNA sequences containing the 3'- and 5'-terminal parts of the B.subtilis chromosomal amyE gene, res pectively; Km, kanamycin resistance gene; spoVG, ribosomal binding site of the spoVG gene of B.subtilis; LacZ, l acZ gene encoding ß-galactosidase; Ap, ampicillin resistance gene. The positions of the restriction sites used are indicated. The lower part of Fig. 4A shows the regions containing P $_1$ (pBTK6) and P_1* (pBTK7) which were inserted in pBTK2. The direction of transcription is indicated with arrowheads. The inserts are not presented to scale. (B) Expression of ß-galactosidase activity of the P $_1$-lacZ (″) and P_1*-lacZ (#) transcriptiona l fusions in B.subtilis 8GK6 and 8GK7 during growth in TY medium. At the times indicated samples were withdrawn from growing cultures for the determination of ß-galactosidas e activit ies. Time zero indicates the end of the exponential growth phase. The time scale is in hours and ß-galactosidase activities are expressed in Miller units per OD600 of the cell cultures. Similarly, the ß - galactosidase activity o f strain 8GK2 (integrated vector pBTK2) was measured as a control. The maximum ß-galactosidase activity measured for the latter strain was 2 Miller units (data not shown).

context (pMV158) it drives transcription of RNAI; (ii) the activity of P_1 increases during the post-exponential growth phase; and (iii) the alterations in P_1* almost completely abolish promoter activity.

We next addressed the question whether P_1 promoter activity is required for the RecA-mediated pathway of pMV158 lagging strand synthesis. Since the P_1 promoter is temporally regulated (Fig. 4B), we analyzed ssDNA accumulation at different growth stages. Total DNA extracts were prepared as a function of time from growing PSL161 and PSL169 cells, each harbouring either pMV(U^-A^-)Em, pLW7 (mutated P_1*), or pLW5 (P_1 and putative

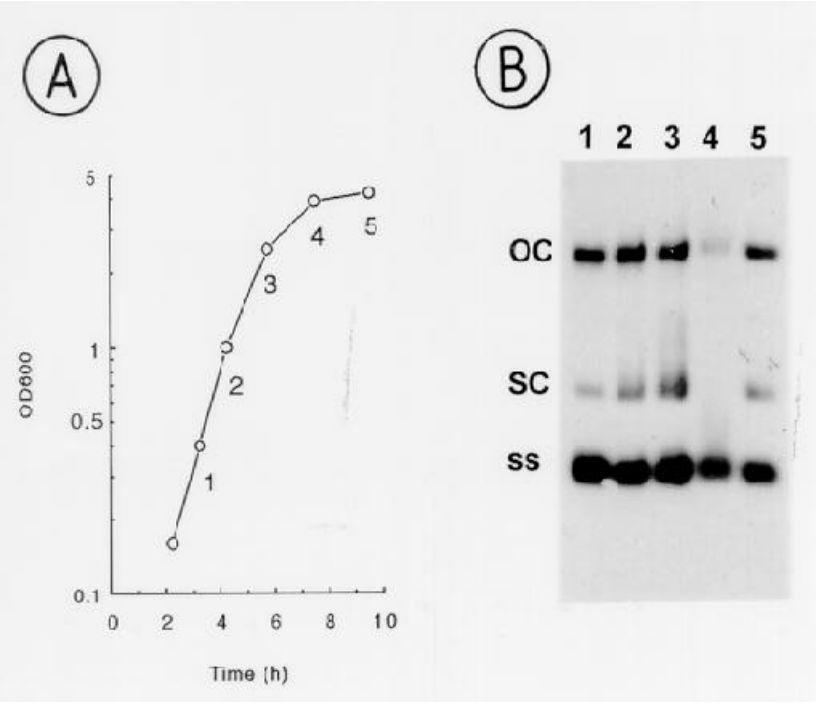

Figure 5. Southern hybridizations of total DNA preparations from B.subtilis PSL169 cells harbouring pMV(U⁻A⁻)Em isolated at different growth phases. (A) Growth curve of the cells and the optical densities (OD600) at the times of sampling. (B) Lanes 1 through 5 represent samples isolated from cells at the times indicated in the growth curve. The transition from the exponential to the stationary growth phase corresponds to about 5-6 hours after the start of the experiment. The blot was hybridized with pMV(U⁻A⁻)Em DNA as a probe. The positions of the various plasmid forms are indicated: single-stranded (ss), supercoiled (sc), and open circular (oc).

RNAI-specifying region deleted). Similar amounts (60 ng) of DNA extracts were used for agarose gel electrophoresis and subsequent Southern hybridizations. The results are shown in Figs. 5 and 6. Irrespective of the growth phase, large amounts of ssDNA were detected with pMV(U⁻A⁻)Em, pLW5 and pLW7 isolated from the RecA-deficient strain PSL169. pMV(U⁻A⁻)Em is shown as an example; pLW5 and pLW7 gave similar results (Fig. 5).

The results of Southern hybridizations obtained with the corresponding DNA extracts from the RecA-proficient strain PSL161, shown in Fig. 6, revealed the following: First, as described in a foregoing section, ssDNA accumulation with pMV(U⁻A⁻)Em was considerably less than with pLW5, in particular during the exponential growth phase. Second, as with pLW5, which lacks the P_1 promoter region, large amounts of ssDNA were present with pLW7 during the exponential growth phase. The latter plasmid contains the inactivated promoter P_1*. Taken together, these results show that during the exponential growth phase conversion of ssDNA of derivatives of pMV158 lacking a functional SSO occurs rather efficiently provided that a functional RecA protein is present and the plasmid contains an active P_1 promoter.

Three striking differences between the plasmid profiles isolated from the RecA-proficient and RecA-deficient strains were observed: (i) in the RecA-proficient strain the amounts of ssDNA of all three plasmids tested decreased drastically after the transition from the exponential to the stationary phase of growth; (ii) the amounts

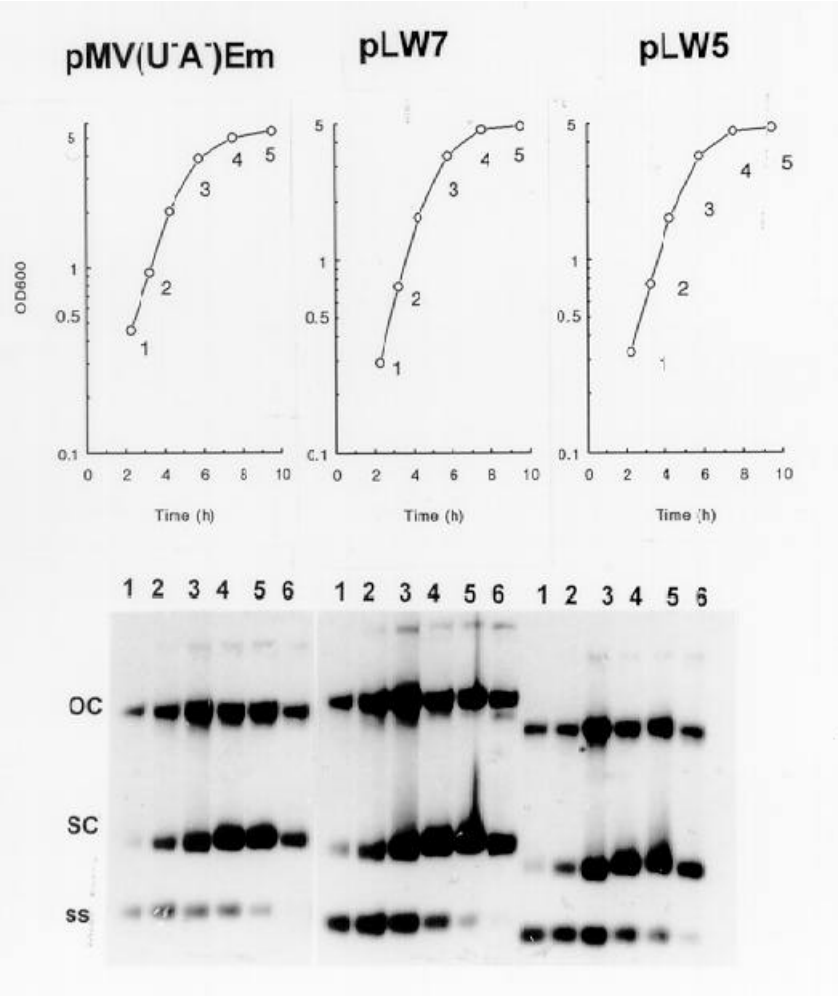

Figure 6. Southern hybridizations of total DNA preparations from B.subtilis PSL161 cells harbouring various pMV(U⁻A⁻)Em derivatives. (A) PSL161 harbouring pMV(U⁻A⁻)Em; (B) PSL161 harbouring pLW7; and (C) PSL161 harbouring pLW5. The upper part of the figure shows the growth curves of the cells and the times of sampling. Time is indicated in hours. OD600 values at the times of sampling are indicated. The lower part of the figure shows the Southern hybridizations of the different samples drawn at the times indicated in the growth curves. Each sixth sample was isolated from an overnight culture. The blots were hybridized with pMV(U⁻A⁻)Em DNA as a probe. The positions of the various plasmid forms are indicated: single-stranded (ss), supercoiled (sc), and open circular (oc).

of double-stranded plasmid forms were relatively low at the early stages of exponential growth (iii), the amounts of the double-stranded plasmid forms were, irrespective of the plasmid tested, higher in the RecA-proficient than in the RecA-deficient background.

DISCUSSION

The major pathway for ssDNA conversion of RCM plasmids from Gram-positive bacteria requires a functional SSO (del Solar et al., 1987; Gruss et al., 1987; Viret and Alonso, 1988; Boe et al., 1989; Bron, 1990; Meijer et al., 1995c; Meijer et al., 1995b). However, even in the absence of functional SSOs replication of RCM plasmids usually still proceeds, also in non-native hosts. This indicates that ssDNA conversion operates via an alternative pathway. In the present work we used pMV158 derivatives lacking a functional SSO to analyze this pathway. The alternative pathway is less effective than

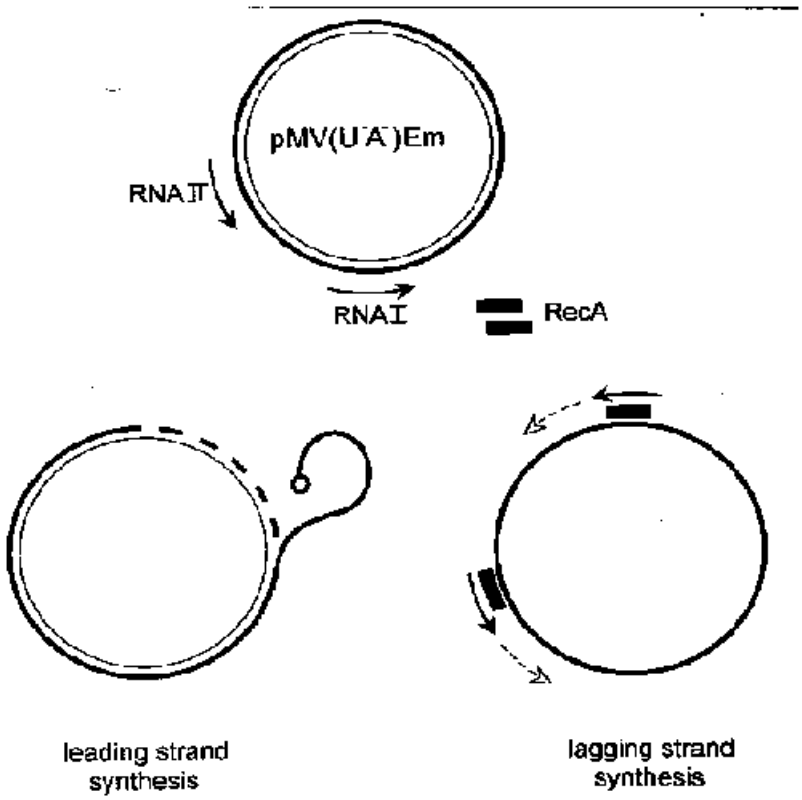

Figure 7. Model for the RecA/RNA-mediated SSO-independent pathway for pMV158 lagging strand synthesis.

Plasmid pMV(U⁻A⁻)Em specifying the RNAI and RNAII molecules which are complementary to the displaced ss plasmid DNA are shown in the upper part of the figure. The lower part shows the leading strand synthesis (left) and the RecA/RNAI-mediated SSO-independent lagging strand synthesis (right). Heavy lines, leading strand; thin lines, lagging strand; continuous lines, parental DNA; interrupted lines, newly synthesized DNA; short arrows, plasmid-specified RNAI molecules; ", Rep protein; ####, RecA protein.

the route via SSOs as is evident from the observation that, unlike in the SSO-dependent pathway, substantial amounts of ssDNA are normally detectable in the absence of an SSO.

Major outcomes of the present work are: (i) the host-encoded RecA protein of B.subtilis is involved in the alternative ssDNA conversion pathway; and (ii) activity of the P_1 promoter, specifying a putative small RNA transcript, also greatly stimulates ssDNA conversion in this pathway; (iii) when the major SSO-dependent ssDNA conversion pathway is operative the effects of the alternative pathway are non-detectable. Based on these results, we propose the following model for the alternative ssDNA conversion pathway (Fig. 7). Plasmid-encoded RNA molecules that are complementary to the ssDNA (e.g. RNAI) anneal in a RecA-stimulated process to the ss plasmid DNA. Analogous to the situation in the SSO-mediated pathway, we envisage that the annealed RNA molecule functions as a primer for lagging strand synthesis. A key element in this model is the capacity of RecA to stimulate the formation of RNA-DNA hybrids. Kirkpatrick and Radding (1992) have shown that, in vitro, the E.coli RecA protein can indeed stimulate the specific annealing

of RNA to complementary ssDNA sequences, resulting in RNA-DNA hybrids. Since the RecA enzymes of E.coli and B.subtilis are structurally (Stranathan et al., 1990) and functionally similar (de Vos et al., 1983; Lovett and Roberts, 1985; Love and Yasbin, 1986; Marrero and Yasbin, 1988), we consider it likely that the RecA protein of B.subtilis is also capable of performing this RNA/DNA annealing activity.

Which plasmid-specified RNA molecules could initiate the postulated alternative conversion of ssDNA? Only RNA molecules which are transcribed from the pMV158 strand which becomes displaced during RCM, such as the small RNAI and RNAII molecules, are candidates for the annealing to the ssDNA replication intermediates. Promoter P_1 has the consensus sequence for major vegetative B.subtilis promoters and could drive the synthesis of a small RNA molecule (approximately 70 nt). This RNA has indeed been demonstrated in exponentially growing cells of Streptococcus pneumoniae and mutations affecting the -10 sequences of the P_1 promoter abolishes RNAI synthesis (P. Acebo, G. del Solar and M. Espinosa, personal communication). So far, no biological function has been assigned to this small RNA. Our results clearly indicated that at least RNAI stimulates the RecA-mediated ssDNA conversion of pMV158 derivatives lacking a functional SSO and, therefore, it seems likely that this small RNA molecule can function as a primer for lagging strand synthesis as proposed in Fig. 7.

Even when the formation of RNAI was prevented, plasmid derivatives lacking SSOs could still establish themselves, but also in this case the relative amounts of ssDNA were lower in a functional RecA background compared to a non-functional RecA background. From these results we infer that, in addition to RNAI, other complementary RNA molecules can initiate ssDNA conversion. A candidate for this initiation is the antisense RNAII. So far, we have not tested this possibility, because manipulation of RNAII expression will interfere with the plasmid copy number control system and, therefore, probably with the levels of ssDNA in a manner independent of the conversion efficiencies of ssDNA.

In RecA-deficient backgrounds, large amounts of ssDNA were detected in each growth phase with all plasmids lacking a functional SSO. However, in the stationary growth phase hardly any ssDNA was detected with the same plasmids in RecA-proficient backgrounds. We consider it unlikely that a single-strand-specific DNase can account for this difference between RecA-proficient and RecA-deficient strains, since such a DNase would have to become active in a RecA-dependent way in the stationary growth phase. To our knowledge, such a DNase is not known. We rather favor the alternative explanation that in the stationary phase all the ssDNA has been converted to duplex plasmid molecules. Presumably, the following process accounts for the observation that in RecA-proficient strains little ssDNA could be detected during the post-exponential phase of growth: During this growth phase cell divisions have nearly stopped and, therefore, the plasmid copy control system will slow down the frequency of replication initiation. As a consequence, reduced amounts of ssDNA will be formed. Since the amounts of ssDNA detected in our assays will be the net result of de novo synthesis and conversion to duplex DNA, this may account for the low levels of ssDNA observed in the post-exponential phase. The decrease in ssDNA accumulation in the RecA-proficient background was not related to the increased P_1 promoter activity during the post-exponential growth phase, since also with pLW5 and pLW7, lacking an active P_1 promoter, ssDNA accumulation decreased

during this phase.

The accumulation of large amounts of ssDNA of RCM plasmids, perhaps in conjunction with the reduced plasmid copy numbers, generally interferes with the maintenance of these plasmids under non-selective growth-conditions (Gruss et al., 1987; del Solar et al., 1987; Bron et al., 1988; Bron, 1990; Bron et al., 1991b; Meijer et al., 1995b). Observations with pMV158 derivatives lacking SSO activity are in accordance with this general observation, at least in the RecA-deficient strains 7G224 (Meijer et al., 1995b) and PSL1 (this study, results not shown). Also pC194, another RCM plasmid which lacks a functional SSO for B.subtilis, is maintained highly unstable in a RecA-deficient strain but not in an isogenic RecA wild-type strain (Alonso et al., 1987).

The importance of the RecA protein for the replication of pMV158 derivatives lacking a functional SSO for B.subtilis may well be extended to other RCM plasmids. Viret and Alonso (1988) reported that pUB110 derivatives lacking its functional palU SSO accumulated more ssDNA in a recA4 strain compared to a wild-type strain. Boe et al. (1989) reported that ssDNA conversion of pUB110 derivatives lacking a functional SSO depends on the host-encoded RNAP. Taking into account that pUB110 is likely to encode a countertranscript RNA for the regulation of its copy number (Maciag et al., 1988), we consider it likely that RecA-mediated annealing of complementary RNA may also constitute an alternative pathway for lagging strand synthesis of pUB110.

It is conceivable that the postulated alternative pathway of ssDNA conversion is involved in the broad-host-range nature of pMV158 and possibly other RCM plasmids. This idea is based on the following considerations. First, although pMV158 is able to replicate in wild-type E.coli cells, it does not replicate in RecA mutants of this bacterium (Lacks et al., 1986). Second, del Solar et al. (1987) showed that, whereas a pMV158 derivative, pLS5, containing palA but lacking palU and the P_1 promoter region, could be established in E.coli, a deletion derivative of pLS5, in which also palA is deleted, could not. Third, de Vos and Simons (1994) reported that pWV01, a broad-host-range plasmid of lactococcal origin which is related to pMV158 (del Solar et al., 1993b) and lacks an efficient SSO for E.coli, transformed E.coli RecA mutants only at very low frequencies. The rare transformants obtained had decreased plasmid copy numbers and accumulated large amounts of ssDNA. These results also indicate that inefficient ssDNA conversion may be a frequent bottleneck in the establishment of a plasmid in a cell.

In summary, in this work we have provided evidence for an alternative pathway for the conversion of ssDNA of RCM plasmids to duplex DNA through RNA/DNA hybridization catalyzed by RecA. This pathway is important for RCM plasmids lacking a functional SSO. In addition to ssDNA conversion, this pathway affects plasmid copy number and plasmid stability, and may well be involved in te determination of the host-range of RCM plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids and media . Bacterial strains and plasmids used are listed in Table 1. TY medium, used for culturing E.coli and B.subtilis, contained Bacto tryptone (1%), Bacto yeast extract (0.5%)

Table 1. Bacterial strains and plasmids

Strains	Relevant properties	Reference
B.subtilis		
PSL1	leuA8 arg15 thrA recA4 $r^-_M m^-_M$	Ostroff and Pène, 1983
PSL161	PSL1 harbouring pBT61, RecA-proficient	This study
PSL169	PSL1 harbouring pBT69K, inactive RecA	This study
8G5	trpC2 tyr1 met his ura nic ade rib	Bron and Venema, 1972
8GK2	lacZ amy^- Km^R	This study
8GK6	P_1-lacZ amy^- Km^R	This study
8GK7	P_1*-lacZ amy^- Km^R	This study
E.coli		
JM83	F- ara △(lac-proAB) rpsL (Str^r) (Φ80d △(lacZ)M15)	Vieira and Messing, 1982
TG90	F- △lacU169 araD139 thiA rpsL relA zad::Tn10 $pcnB^+$	Lopilato et al., 1986
Plasmids	**Properties**	**Reference**
pMV(U^-A^-)Em	broad-host-range, high-copy pMV158 derivative lacking palA and palU, Em^R	Meijer et al., 1995b
pKM1	pUC7 derivative carrying the Km^R gene from the Streptococcus faecalis plasmid pJH1, Amp^R, Km^R	Kiel et al., 1987
pLW5	pMV(U^-A^-)Em derivative lacking the 0.36 kb region comprizing P_1 and RNAI	This study
pLW6	pMV(U^-A^-)Em derivative containing a unique XbaI site downstream of P_1	This study
pLW7	pLW6 derivative in which P_1 was mutated	This study
pBT61	pUB110 cop1 derivative, contains B.subtilis recA gene, Km^R	Gassel and Alonso, 1989
pBT69K	pBT61 derivative, contains kanamycin marker inserted in recA gene, Km^R	Haijema et al., 1995
pE1	amyE-lacZ insertion vector, Amp^R, Cm^R	Haijema et al., 1995
pBTK2	amyE-lacZ insertion vector, pE1 derivative, Amp^R, Km^R	This study
pBTK6	pBTK2 derivative, contains transcriptional P_1-lacZ fusion, Amp^R, Km^R	This study
pBTK7	pBTK2 derivative, contains transcriptional P_1*-lacZ fusion, Amp^R, Km^R,	This study

Table 2. Primers used

Primer	position #	Sequence (5' → 3')
LW20	359 ←	GCCAT GGTACCCGG [1]GAGCTCGAATTCCCG
LW21	171 ←	GTGCTTTTAGTTCC TCtaGA [2]ACTTTTAGCGTC
LW22	1 →	CTCAAA CGCGC [3]CCTTTGAGCTATCCGT
LW23	139 ←	GCATATTATATCATGGGGCGAGAAATTC actagt [4]AATAATG

The following restriction sites are underlined 1: Kpn I/Hpa II; 2: Xba I; 3: Cfo I/Mvn I; 4: Spe I. # Numbers indicates the start positions of the primers according to Fig. 1B. Nucleotides in the mutation primers that deviate from the original pMV(U⁻A⁻)Em sequence are shown in lowercase letters.

and NaCl (1%). Solid media contained, in addition, 2% agar. Erythromycin was added to final concentrations of 4 and 150 µg/ml for B.subtilis and E.coli, respectively. Final concentrations of 50 µg/ml were used for the selection on kanamycin of B.subtilis and ampicilline of E.coli.

DNA techniques. All DNA manipulations were carried out according to Sambrook et al. (1989). Restriction enzymes were used as indicated by the suppliers. Plasmid DNA was isolated by the alkaline-lysis method as described (Sambrook et al., 1989). Total DNA extracts from cells were prepared as described before (Meijer et al., 1995c). DNA fragments were isolated from gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, USA). Measurements of DNA concentrations in total DNA extracts were determined using the TK0 100 mini fluorometer (Hoefer Scientific Instruments, San Francisco, USA). Agarose gels used for Southern hybridizations were treated as follows: (i) 15 min irradiation on a UV-transilluminator; (ii) 20 min incubation in 0.4 M NaOH/0.6 M NaCl; followed by (iii) 20 min incubation in 1.5 M NaCl/0.5 M Tris-HCl (pH 7.5) buffer. DNAs were blotted onto GeneScreen Plus filters (Dupont NEN, Boston, Ma) using 1.5 M NaCl/0.15 M tri-sodium citrate as transfer buffer. Probe labeling, DNA hybridization conditions and washing steps were performed using the enhanced chemiluminescence DNA labeling and detection system (Amersham International plc, Amersham, UK).

Transformation of B.subtilis and E.coli. Competent cells and protoplasts of B.subtilis were prepared and transformed as described (Bron, 1990). $CaCl_2$-treated E.coli cells were transformed as described (Sambrook et al., 1989).

PCR techniques. PCR was carried out essentially as described by Innis and Gelfand (1980). The proofreading-proficient Vent DNA polymerase (New England Biolabs, Beverly, Ma, USA) was used throughout. Template DNAs were denatured for 1 min at 94 °C. Next, DNA fragments were amplified in 30 cycles of denaturation (30 sec; 94°C), primer annealing (1 min; 50°C), and DNA synthesis (3 min; 73°C). Primers used are listed in Table 2.

ß-Galactosidase activity assays. Overnight cultures were diluted hundred fold into fresh medium and samples were taken at fourty-five minute intervals for optical density readings (OD600). ß-Galactosidase activity assays (expressed as units per OD600) were carried out as described by Miller et al. (1982).

Construction of plasmids and strains

used. Strain PSL161 is strain PSL1 containing plasmid pBT61; strain PSL169 is PSL1 containing plasmid pBT69K. Plasmid pBT61 is a low-copy number derivative of pUB110 (pUB110cop1) containing the B.subtilis recA gene (Gassel and Alonso, 1989). pBT69K is a derivative of pBT61 (Haijema et al., 1995), in which the recA gene is inactivated by the insertion of the kanamycin resistance (Km^R) gene of pKM1. Of the three PSL strains used, only PSL161 was resistant to mitomycin C, indicating that this strain produces active RecA protein.

The construction of pMV(U^-A^-)Em, a pMV158 derivative lacking its SSOs, has been described before (Meijer et al., 1995c). A map of pMV(U^-A^-)Em and the relevant features of pLW5, pLW6, and pLW7 are shown in Fig. 1A. pLW5, lacking the region encoding RNAI (including promoter P_1), was constructed as follows: after digestion of pMV(U^-A^-)Em with CfoI and HpaII, the largest restriction fragment was isolated, treated with T4-DNA polymerase and selfligated. pLW6, in which an extra TA dinucleotide was inserted 20 bp downstream of the -10 promoter sequence of P_1, resulting in a unique XbaI site, was constructed in several steps. First, a PCR was performed using primers LW22 and LW21 (mutation primer) and pMV(U^-A^-)Em as template DNA. The resulting 172 bp PCR product was used as a primer, together with primer LW20, in a second PCR with pMV(U^-A^-)Em as template DNA. The resulting PCR product (359 bp) was digested with KpnI and CfoI, and used to replace the corresponding KpnI/CfoI fragment of pMV(U^-A^-)Em. pLW7, in which specifically P_1 is altered, was constructed using a strategy analogous to that used for pLW6. The first PCR was performed using the primers LW22 and LW23 (mutation primer) and pLW6 as template DNA. The resulting PCR product (138 bp) was used as a primer, together with primer LW20, in a second PCR using pLW6 as template DNA. The resulting PCR product (359 bp) was digested with KpnI and CfoI and ligated to the large KpnI/CfoI fragment of pMV(U^-A^-)Em. Ligation mixtures for the construction of pLW6 and pLW7 were used to transform competent E.coli JM83 cells. Plasmid DNA from erythromycin-resistant colonies was isolated and analyzed for the presence of XbaI (pLW6) or SpeI sites (pLW7). DNA sequence analysis was used to verify that the sequences of the PCR-amplified regions in representative clones of pLW6 and pLW7 were correct.

pBTK2 and derivatives thereof were used to monitor promoter activities of the intact (pLW6) and the mutated P_1 (pLW7) by assaying ß-galactosidase activity. A schematic representation of pBTK2 is shown in Fig. 4A. The latter plasmid was derived from pE1 (Haijema et al., 1995). pE1 is a pBR322-based integration vector which carries the amyE-lacZ region. In pE1, the efficient translational start signals of the B.subtilis gene spoVG are present upstream of the promoterless E.coli lacZ gene. pBTK2 is pE1 in which the 1.1 kb StuI/SmaI fragment containing the chloramphenicol resistance (Cm^R) marker was replaced by the 1.4 kb HindII fragment of pKM1 containing the kanamycin resistance (Km^R) marker (Fig. 1B, upper part). The pBTK2-derived plasmids pBTK6 and pBTK7 were constructed as follows: the 150 bp MvnI/XbaI fragments of pLW6 (intact P_1) and pLW7 (mutated $_1P$) were ligated into the SmaI/XbaI sites of pBTK2 (Fig. 4A, lower part). The desired clones were checked by restriction analysis. Plasmid pBTK6 could only be obtained from E.coli TG90 cells in which pBR322 derivatives are maintained at a low plasmid copy number (Lopilato et al., 1986). Following linearization with ScaI, plasmids pBTK2, pBTK6 and pBTK7 were integrated into the amyE locus of strain 8G5 by replacement recombination resulting in strains 8GK2, 8GK6 and 8GK7,

respectively.

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REFERENCES

Alonso, J.C., Viret, J., and Tailor, R.H. (1987). Plasmid maintenance in Bacillus subtilis recombination-deficient mutants. Mol. Gen. Genet. 208: 349-352.

Birch, P. and Khan, S. (1992). Replication of single-stranded plasmid pT181 DNA in vitro. Proc. Natl. Acad. Sci. USA 89: 290-294.

Boe, L., Gros, M., te Riele, H., Ehrlich, S.D., and Gruss, A. (1989). Replication origins of single-stranded-DNA plasmid pUB110. J. Bacteriol. 171: 3366-3372.

Bron, S., Haima, P., Van Belkum, M.J., and Luxen, E. (1988). Segregational plasmid instability in Bacillus subtilis. In Genetics and Biotechnology of Bacilli. Ganesan, A.T. and Hoch, J.A. (eds.) San Diego: Academic Press, Inc. pp. 305-309.

Bron, S. (1990). Plasmids. In Molecular Biological Methods for Bacillus. Harwood, C.R. and Cutting, S.M. (eds.) Chichester, UK: John Wiley & Sons Ltd. pp. 75-174.

Bron, S., Holsappel, S., Venema, G., and Peeters, B.P.H. (1991a). Plasmid deletion formation between short direct repeats in Bacillus subtilis is stimulated by single-stranded rolling-circle replication intermediates. Mol. Gen. Genet. 226: 88-96.

Bron, S., Meijer, W., Holsappel, S., and Haima, P. (1991b). Plasmid instability and molecular cloning in Bacillus subtilis. Res. Microbiol. 142: 875-883.

Bron, S. and Venema, G. (1972). Ultraviolet inactivation and excision repair in Bacillus subtilis. I. Construction and characterization of a transformable eightfold auxotrophic strain and two ultraviolet-sensitive derivatives. Mutat. Res 15: 1-10.

Ceglowski, P., Lüder, G., and Alonso, J.C. (1990). Genetic analysis of recE activities in Bacillus subtilis. Mol. Gen. Genet. 224: 441-445.

de Vos, W.M., de Vries, S.C., and Venema, G. (1983). Cloning and expression of the Escherichia coli recA gene in Bacillus subtilis. Gene 25: 301-308.

de Vos, W.M. and Simons, G.F.M. (1994). Gene cloning and expression systems in Lactococci. In Genetics and biotechnology of lactic acid bacteria. Gasson, M.J. and de Vos, W.M. (eds.) Glasgow: Blackie Academic and Professional, pp. 52-105.

del Solar, G., Kramer, G., Ballester, S., and Espinosa, M. (1993a). Replication of the promiscuous plasmid pLS1: a region encompassing the minus origin of replication is associated with stable plasmid inheritance. Mol. Gen. Genet. 241: 97-105.

del Solar, G., Moscoso, M., and Espinosa, M. (1993b). Rolling circle-replicating plasmids from gram-positive and -negative bacteria: a wall falls. Mol. Microbiol. 8: 789-796.

del Solar, G.H., Puyet, A., and Espinosa, M. (1987). Initiation signals for the conversion of single stranded to double stranded DNA forms in the streptococcal plasmid pLS1. Nucleic Acids Res. 15: 5561-5580.

del Solar, G.H. and Espinosa, M. (1992). The copy number of plasmid pLS1 is regulated by two trans-acting plasmid products: The antisense RNA II and the repressor protein, RepA. Mol. Microbiol. 6: 83-94.

Dempsey, L.A., Zhao, A.C., and Khan, S.A. (1995). Localization of the start sites of

lagging-strand replication of rolling-circle plasmids from Gram-positive bacteria. Mol. Microbiol. 15: 679-687.

Ehrlich, S.D., Noirot, P., Petit, M.A., Jannière, L., Michel, B., and te Riele, H. (1986). Structural instability of Bacillus subtilis plasmids. In Genetic engineering. Setlow, J.K. and Hollaender, A. (eds.) New York: Plenum Publishing Corp. pp. 71-83.

Gassel, M. and Alonso, J.C. (1989). Expression of the recE gene during induction of the SOS response in Bacillus subtilis recombination deficient strains. Mol. Microbiol. 3: 1269-1276.

Gruss, A. and Ehrlich, S.D. (1989). The family of higly interrelated single-stranded deoxyribonucleic acid plasmids. Microbiol. Rev. 53: 231-241.

Gruss, A.D., Ross, H.F., and Novick, R.P. (1987). Functional analysis of a palindromic sequence required for normal replication of several staphylococcal plasmids. Proc. Natl. Acad. Sci. USA 84: 2165-2169.

Haijema, B., Hamoen, L.W., Venema, G., and van Sinderen, D. (1995). Expression of the ATP-dependent deoxyribonuclease of Bacillus subtilis is under competence-mediated control. Mol. Microbiol. 15: 203-211.

Innis, M.A. and Gelfand, D.H. (1980). Optimization of PCRs. In PCR protocols: a guide to methods and applications. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds.) Academic Press,

Jannière, L., Gruss, A., and Ehrlich, S.D. (1993). Plasmids. In Bacillus subtilis and other Gram-positive bacteria; Biochemistry, physiology and molecular genetics. Sonenshein, A.L., Hoch, J.A., and Losick, R. (eds.) Washington: American Society for Microbiology, pp. 625-644.

Kiel, J.A.K.W., van der Vossen, J.P.M.J., and Venema, G. (1987). A general method for the construction of Escherichia coli mutants by homologous recombination and plasmid segregation. Mol. Gen. Genet. 207: 294-301.

Kirkpatrick, D.P., Jagadeeshwar, R., and Radding, C.M. (1992). RNA-DNA hybridization promoted by E.coli RecA protein. Nucleic Acids Res. 20: 4339-4346.

Kramer, M.G., del Solar, G., and Espinosa, M. (1995). Lagging-strand origins of the promiscuous plasmid pMV158: physical and functional characterization. Microbiology 141: 655-662.

Lacks, S.A., Lopez, P., Greenberg, B., and Espinosa, M. (1986). Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. J. Mol. Biol. 192: 753-765.

Leonhardt, H. (1990). Identification of a low-copy-number mutation within the pUB110 replicon and its effect on plasmid stability in Bacillus subtilis. Gene 94: 121-124.

Lopilato, J., Bortner, S., and Beckwith, J. (1986). Mutations in a new chromosomal gene of Escherichia coli K-12, pcnB, reduce plasmid copy number of pBR322 and its derivatives. Mol. Gen. Genet. 205: 285-290.

Love, P.E. and Yasbin, R.E. (1986). Induction of the Bacillus subtilis SOS-like response by Escherichia coli RecA protein. Proc. Natl. Acad. Sci. USA 83: 5204-5208.

Lovett, C.M. and Roberts, J.W. (1985). Purification of a RecA protein analogue from Bacillus subtilis. J. Biol. Chem. 260: 3305-3313.

Maciag, I.E., Viret, J., and Alonso, J.C. (1988). Replication and incompatibility properties of plasmid pUB110 in Bacillus subtilis. Mol. Gen. Genet. 212: 232-240.

Marrero, R. and Yasbin, R. (1988). Cloning of the Bacillus subtilis $recE^+$ gene and functional expression of $recE^+$ in B.subtilis. J. Bacteriol. 170: 335-344.

Meijer, W.J.J., van der Lelie, D., Venema, G., and Bron, S. (1995a). Effects of the generation of single-stranded DNA on the maintenance of plasmid pMV158 and derivatives in Lactococcus lactis. Plasmid 33: 91-99.

Meijer, W.J.J., van der Lelie, D., Venema, G., and Bron, S. (1995b). Effects of the generation of single-stranded DNA on the maintenance of plasmid pMV158 and derivatives in different Bacillus subtilis strains. Plasmid 33: 79-89.

Meijer, W.J.J., Venema, G., and Bron, S. (1995c). Characterization of single strand origins of cryptic rolling-circle plasmids from Bacillus subtilis. Nucleic Acids Res. 23: 612-619.

Miller, J.H. (1982). Experiments in molecular

genetics Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Novick, R.P. (1989). Staphylococcal plasmids and their replication. Annu. Rev. Microbiol. 43: 537-565.

Ostroff, G.R. and Pène, J.J. (1983). Molecular cloning with bifunctional plasmid vectors in Bacillus subtilis. III. Isolation of a spontaneous mutant of B.subtilis with enhanced transformability for Escherichia coli propagated chimaeric plasmids. J. Bacteriol. 156: 934-936.

Puyet, A., del Solar, G.H., and Espinosa, M. (1988). Identification of the origin and direction of replication of the broad-host-range plasmid pLS1. Nucleic Acids Res. 16: 115-133.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Seery, L. and Devine, K.M. (1993). Analysis of features contributing to activity of the single-stranded origin of Bacillus plasmid pBAA1. J. Bacteriol. 175: 1988-1994.

Stranathan, M.C., Bayles, K.W., and Yasbin, R.E. (1990). The nucleotide sequence of the $recE^+$ gene of Bacillus subtilis. Nucleic Acids Res. 18: 4249

Vieira, J. and Messing, J. (1982). The pUC plasmids, a M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19: 259-268.

Viret, J. and Alonso, J.C. (1988). A DNA sequence outside the pUB110 minimal replicon is required for normal replication in Bacillus subtilis. Nucleic Acids Res. 16: 4389-4406.