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Replication and maintenance of plasmids in Bacillus subtilis

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Chapter V

Characterization of single strand origins of cryptic rolling-circle plasmids from Bacillus subtilis

Wilfried J.J. Meijer, Gerard Venema and Sierd Bron

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SUMMARY

In this chapter we describe the isolation and characterization of single strand origins (SSOs) of several cryptic Bacillus subtilis plasmids which use the rolling-circle mechanism of replication. The plasmids used in this study involved pTA1015, pTA1020, pTA1030, pTA1040, pTA1050 and pTA1060. The SSO of pTA1015 was isolated by shotgun cloning in a specially designed vector, pWM100, which has no SSO of its own. Sequence analysis revealed that the SSO of pTA1015 is almost identical to formerly described palT type SSOs. Also pTA1020 and pTA1060 were shown to contain SSOs highly homologous to palT. Using Southern hybridization with the palT of pTA1015 as a probe, the SSO of pTA1040 was cloned. Sequence analysis revealed a region of 200 bp which is 77% identical to the palT of pTA1015. The plasmids pTA1030 and pTA1050 contain an SSO which is highly homologous to the SSO of pTA1040. The majority of the SSOs of rolling-circle plasmids from B.subtilis seem to belong to two related families which we denote as palT1 (present on pTA1015, pTA1020 and pTA1060) and palT2 (present on pTA1030, pTA1040 and pTA1 050). Both families of SSOs are highly efficient single-strand-conversion signals in B.subtilis.

INTRODUCTION

Many cloning and expression vectors for Bacillus subtilis are based on small multicopy plasmids that use the rolling-circle mechanism (RCM) of replication [for reviews see (Gruss and Ehrlich, 1989; Novick, 1989; Jannière et al., 1993)]. This replication mechanism is characterized by the generation of single stranded (ss) DNA intermediates. Without efficient conversion to duplex plasmid DNA molecules, these ssDNA replication intermediates accumulate in the host cell. A direct relation has been reported between the accumulation of large amounts of ssDNA and both structural and segregational plasmid instability (del Solar et al., 1987; Gruss et al., 1987; Bron et al., 1991; Boe et al., 1989; Viret and Alonso, 1988; Meijer et al., 1995a; Meijer et al., 1995b). Structural instability is most frequently manifested by the formation of deletion derivatives and segregational instability refers to the loss of the plasmid population from the culture. Thus, efficient ssDNA conversion is essential for optimal stability of RCM plasmids. Conversion of ssDNA is initiated from a non-coding plasmid-located region, called single strand origin (SSO). A characteristic feature of SSOs is that these contain regions of dyad symmetry which have a high potential to form imperfect stem-loop structures (Gruss et al., 1987; Boe et al., 1989; Devine et al., 1989; van der Lelie et al., 1989; del Solar et al., 1993). Another feature of SSOs is that they are functional in only one orientation (Gruss et al., 1987; Boe et al., 1989). Based on sequence homologies, most SSOs can be divided in three families: the palA-, palU- and palT types (Gruss et al., 1987; Boe et al., 1989; Devine et al., 1989; van der Lelie et al., 1989; Chang et al., 1987). All of these rely on the host-encoded RNA-polymerase for the priming of the

complementary strand synthesis on the ssDNA template. Whereas palA or palU type SSOs are generally present on Staphylococcus and Streptococcus plasmids, palT type SSOs were identified on the B.subtilis plasmids pLS11 (Chang et al., 1987), pBAA1 (Devine et al., 1989; Seery and Devine, 1993) and pTA1060 (Bron et al., 1987). Recently, additional SSOs have been described which cannot be classified in one of the above-mentioned families. For instance, Leer et al. (1992) described an SSO present on the small Lactobacillus plasmid p8014-2; Madsen et al. (1993) have identified an SSO showing similarity to the palT type SSO on the Bacillus thuringiensis plasmid pTX14-3; and we have studied the SSO from the lactococcal plasmid pWVO1 (palW) (our own unpublished results). The latter two SSOs differ from the others in that the ssDNA conversion is, at least partially, independent of the host-encoded RNA polymerase (Madsen et al., 1993) our own unpublished results).

To identify and characterize additional plasmid-located SSOs, we have developed a vector, pWM100, which is specially designed for (i) the selection of functional SSOs; and (ii) the testing of ssDNA conversion activity of cloned potential SSOs. The main features of pWM100 are: (i) it is based on the streptococcal plasmid pMV158 (Burdett, 1980) which uses the RCM of replication; (ii) it is segregationally highly unstable because of the deletion of the DNA sequences that might exert SSO activity; (iii) it contains convenient multiple cloning sites; and (iv) it enables lacZα-complementation for blue/white screening of recombinant plasmids. Since pWM100 carries the replication functions of the broad-host-range plasmid pMV158, it can be used in Gram-positive as well as Gram-negative hosts. In the present work pWM100 was specifically used for the selection and characterization of SSOs present on cryptic RCM plasmids of B.subtilis. Two families of efficient SSOs are described which are useful for the construction of stable cloning vectors for B.subtilis.

RESULTS

Construction of the pWM100/ B.subtilis BD630M15 host/vector system

A schematic map of pWM100 is shown in Fig. 1. pWM100 is based on a derivative of pMV158, pMV(U$^-$A$^-$)Em, which lacks the palA and palU type SSOs of pMV158 and contains the Em^R gene from pE194 (Meijer et al., 1995b). The region upstream the Em^R gene shows homology with sequences flanking the palU type SSO. Since this might interfere with the structural stability of recombinant plasmids containing palU-like SSOs this 240 bp homology region was replaced by a short linker sequence which introduced unique AvaII, SacI and PvuI sites. Next, the 2.0 kb ScaI/SphI fragment of pGKH2 (Haima et al., 1990) containing the cat86::lacZα fusion was introduced into the SmaI site. The C-terminal part of the cat86 gene, which interferes with lacZα complementation, was deleted and replaced by the pUC9 multiple cloning site resulting in the vector pWM100.

The optimized lacZM15 gene is present on the chromosome of B.subtilis strain 6GM15 (Km^R and Cm^R) (Haima et al., 1990). A disadvantage of this strain is its low

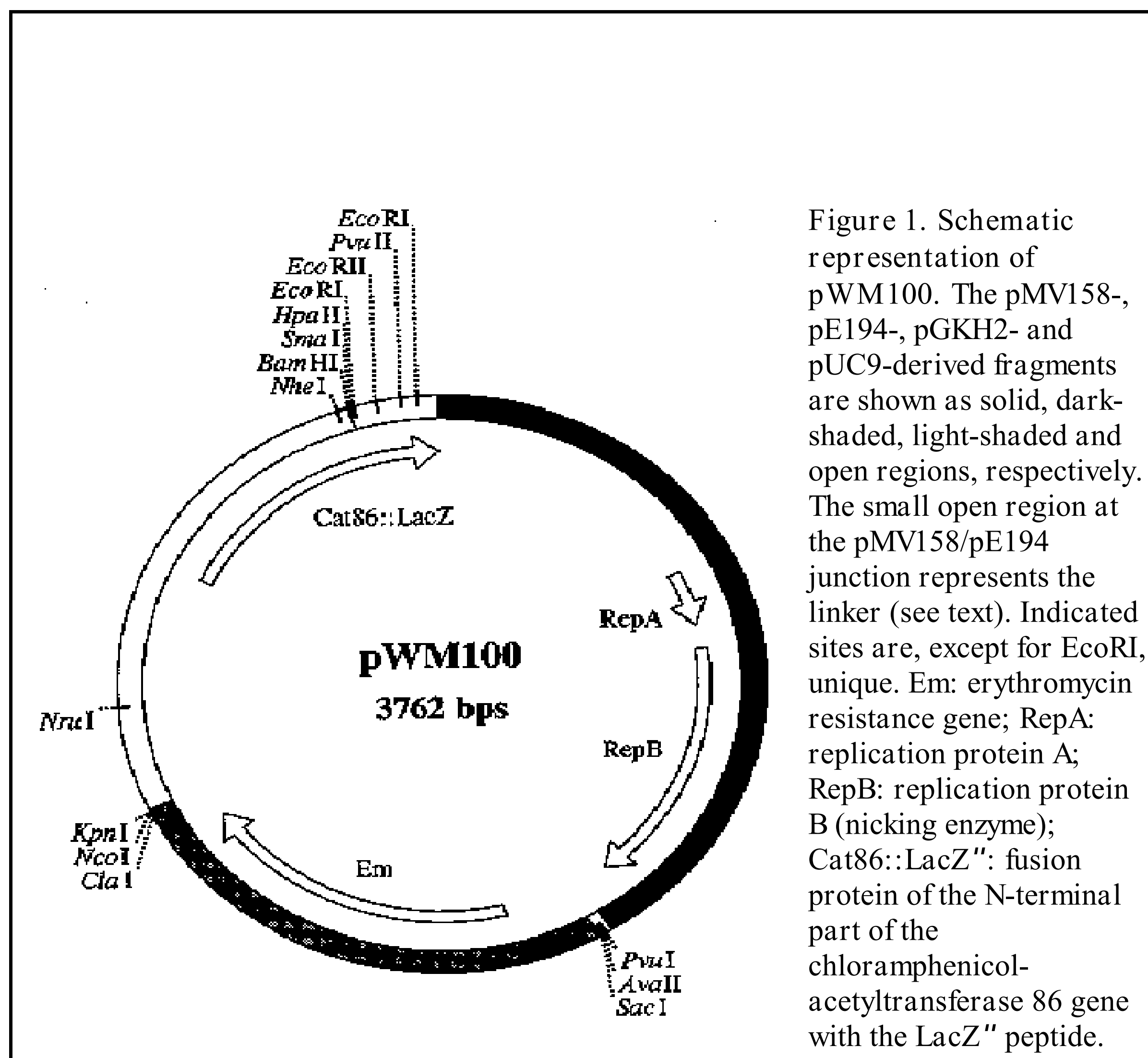

Figure 1. Schematic representation of pWM100. The pMV158-, pE194-, pGKH2- and pUC9-derived fragments are shown as solid, dark-shaded, light-shaded and open regions, respectively. The small open region at the pMV158/pE194 junction represents the linker (see text). Indicated sites are, except for EcoRI, unique. Em: erythromycin resistance gene; RepA: replication protein A; RepB: replication protein B (nicking enzyme); Cat86::LacZ": fusion protein of the N-terminal part of the chloramphenicol-acetyltransferase 86 gene with the LacZ" peptide.

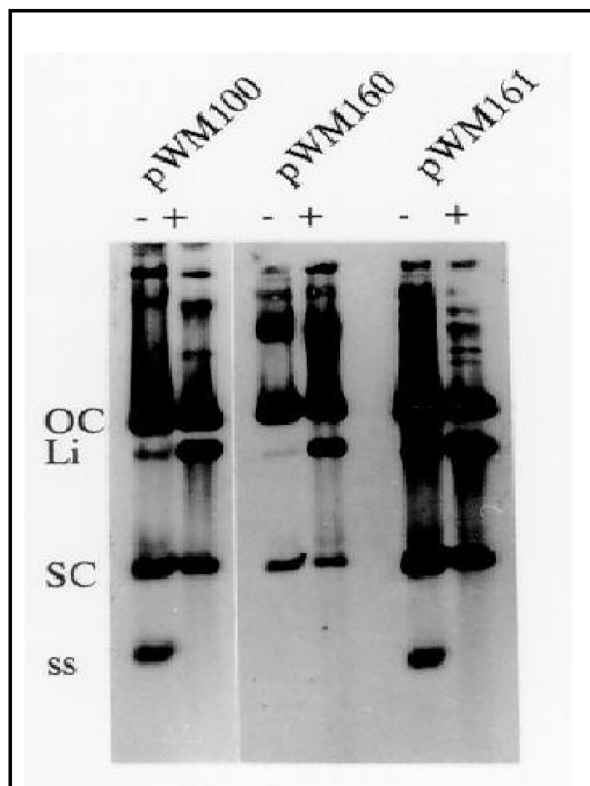

Figure 2. Total DNA lysates of B.subtilis strain BD630M15 harbouring pWM100, pWM160 or pWM161.
Sample s were incubated without (-) or with (+) S1-endonuclease. The positions of the variou s

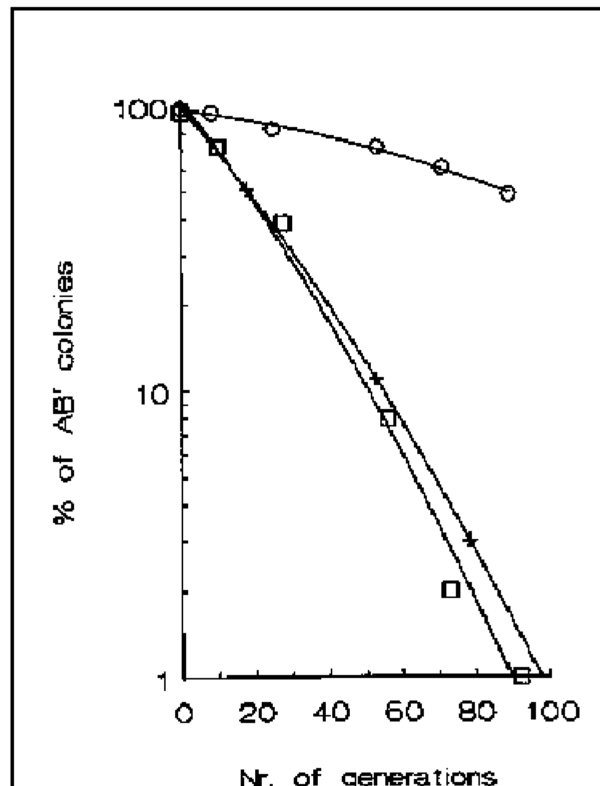

Figure 3. Maintenance of pWM100, pWM160 and pWM161 in B.subtilis BD630M15 under non-selective growth conditions.
O: pWM100; ": pWM160; +: pWM161.

efficiency of protoplasts transformation. Therefore, the lacZM15 gene was transferred to B.subtilis BD630 (Albano et al., 1989) by transforming competent cells of this strain with 6GM15 chromosomal DNA. The resulting strain was designated BD630M15. The pWM100/BD630M15 host/vector system allowed the blue-white assay (ß-galactosidase LacZα complementation) for the selection of recombinant plasmids.

Suitability of pWM100 as selection vector for SSOs

Because pWM100 is devoid of any DNA sequence known to be functional as an SSO, it was expected to accumulate as ssDNA and to be unstably maintained under non-selective growth conditions. To test these predictions, ssDNA accumulation and maintenance of pWM100 were analyzed in logarithmically growing B.subtilis BD630M15 cells. As shown in Figure 2, pWM100 accumulated considerable amounts of ssDNA (pWM160 and pWM161 are described below). Figure 3 shows that pWM100 was indeed maintained unstably.

Next, the feasability of our approach for the selection of functional SSOs was tested. For this purpose two different SSOs, known to function in B.subtilis, were cloned into pWM100 and the resulting constructs were tested for ssDNA accumulation and plasmid maintenance. The SSOs chosen were the palU type SSO of pUB110 (Viret and Alonso, 1988; Boe et al., 1989) and the palT type SSO of pTA1060 (Bron et al., 1987). In order to place suitable restriction sites at its ends, the 546 bp AluI fragment of pTA1060, containing palT, was first

subcloned in both orientations into the unique SmaI site of pUC9. PalT was reisolated from these plasmids as a 560 bp BamHI/EcoRI fragment and cloned between the BamHI/EcoRI sites of pWM100. Using this strategy, palT was cloned in both orientations in pWM100, resulting in pWM160 (functional orientation) and pWM161 (non-functional orientation). Logarithmically growing B.subtilis BD630M15 cells harbouring pWM160 or pWM161 were used to analyze ssDNA accumulation and plasmid maintenance. The results are presented in Figures 2 and 3. Whereas similar amounts of ssDNA were detected with pWM161 and pWM100, no ssDNA was detected with pWM160. This efficient ssDNA conversion of the latter construct was accompanied by a marked increase in plasmid maintenance (Figure 3). Similar results were obtained for palU of pUB110 (results not shown). These results indicate that, in the correct orientation, the presence of a functional heterologous SSO in pWM100 greatly improves its maintenance. This demonstrates that pWM100 can be used to select functional SSOs from plasmids in B.subtilis.

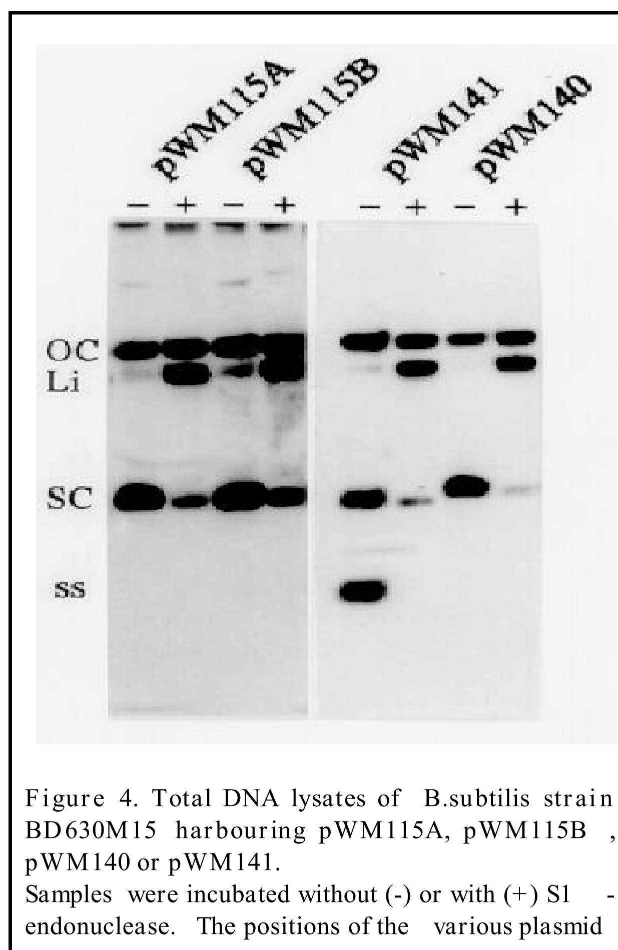

Figure 4. Total DNA lysates of B.subtilis strain BD630M15 harbouring pWM115A, pWM115B , pWM140 or pWM141.
Samples were incubated without (-) or with (+) S1 -endonuclease. The positions of the various plasmid

Cloning of the SSO of the cryptic B.subtilis plasmid pTA1015

Next, pWM100 was used to select the SSO of the cryptic plasmid pTA1015 of B.subtilis IAM1028 (Uozumi et al., 1980). This RCM plasmid was chosen because it is maintained stably in spite of its low copy-number and, therefore, is likely to contain an efficient SSO (Bron et al., 1991). Another reason for choosing pTA1015 is that it is wide-spread among B.subtilis strains (see Discussion).

To clone the SSO of pTA1015, the plasmid was partially digested with AluI and the fragments, ranging from 200 to 1200 bp, were isolated from an agarose gel and cloned in pWM100 linearized with SmaI. To prevent self-ligation, the linearized vector was treated with alkaline phosphatase before the ligation step. The resulting ligation mixture was used to transform B.subtilis BD630M15 protoplasts. Transformants were transferred to TY plates containing Em and XGal. White colonies (248 in total) were pooled and used to inoculate 500 ml of TY medium which was incubated at 37°C. Every 8 hours the growing culture was diluted 10^5 -fold into fresh prewarmed TY medium (100 ml) to maintain logarithmic growth. The culture was alternately grown under non-selective conditions (± 80 generations) and selective conditions (± 10 generations) to enrich for stable clones. After three such

Figure 5. DNA sequence of the 338 bp cloned AluI fragment of plasmid pTA1015 containing its SSO and comparison of this sequence with DNA sequences from pBAA1 (Devine et al., 1989), pUH1 (Hara et al., 1992), pLS11 (Chang et al., 1987), and pTA1060 (our own unpublished results).
The most conserved region common to these sequences (position 75 to 274) is marked with arrows and printed in bold.

cycles of growth, appropriate dilutions of the culture were plated on selective TY agar. Sixteen randomly chosen colonies were analyzed for their plasmid contents by restriction analysis. Ten of these colonies harboured plasmids with inserts which ranged from approximately 300 to 600 bp. Plasmid DNA from the ten colonies was blotted to nitrocellulose membranes and the smallest insert was used as a probe for Southern hybridization. All ten clones gave a hybridization signal, indicating that each of them contained at least the smallest fragment (results not shown). Two clones, denoted pWM115A and pWM115B, that contained the smallest fragment were used for further analysis. Total DNA preparations from logarithmically growing cells containing pWM115A or pWM115B were used for the analysis of ssDNA accumulation by Southern hybridization. Untreated and S1-nuclease-treated samples of the extracts were electrophoresed on an agarose gel, blotted to nylon membranes and hybridized with pWM100 DNA. The results presented in Figure 4 show that neither of the two plasmids accumulated detectable amounts of ssDNA, suggesting that a functional ssDNA conversion signal had been cloned (plasmids pWM140 and pWM141 are described in one of the following sections). Both plasmids were introduced into E.coli and the inserts were sequenced. The sequences of both inserts (338 bp) were identical. The sequence is shown in Figure 5. Comparison of this sequence to known sequences revealed that the pTA1015 fragment is almost identical to the palT type SSOs identified on the cryptic B.subtilis plasmids pLS11 from strain IF03022 (Chang et al., 1987) and pBAA1 from the industrial strain BAA1 (Devine et al., 1989). From our own unpublished results we know that the SSO from plasmid pTA1060 is also of the palT type. Also plasmid pUH1 from an industrial B.subtilis22 natto strain contains a region which is highly homologous to the SSO of

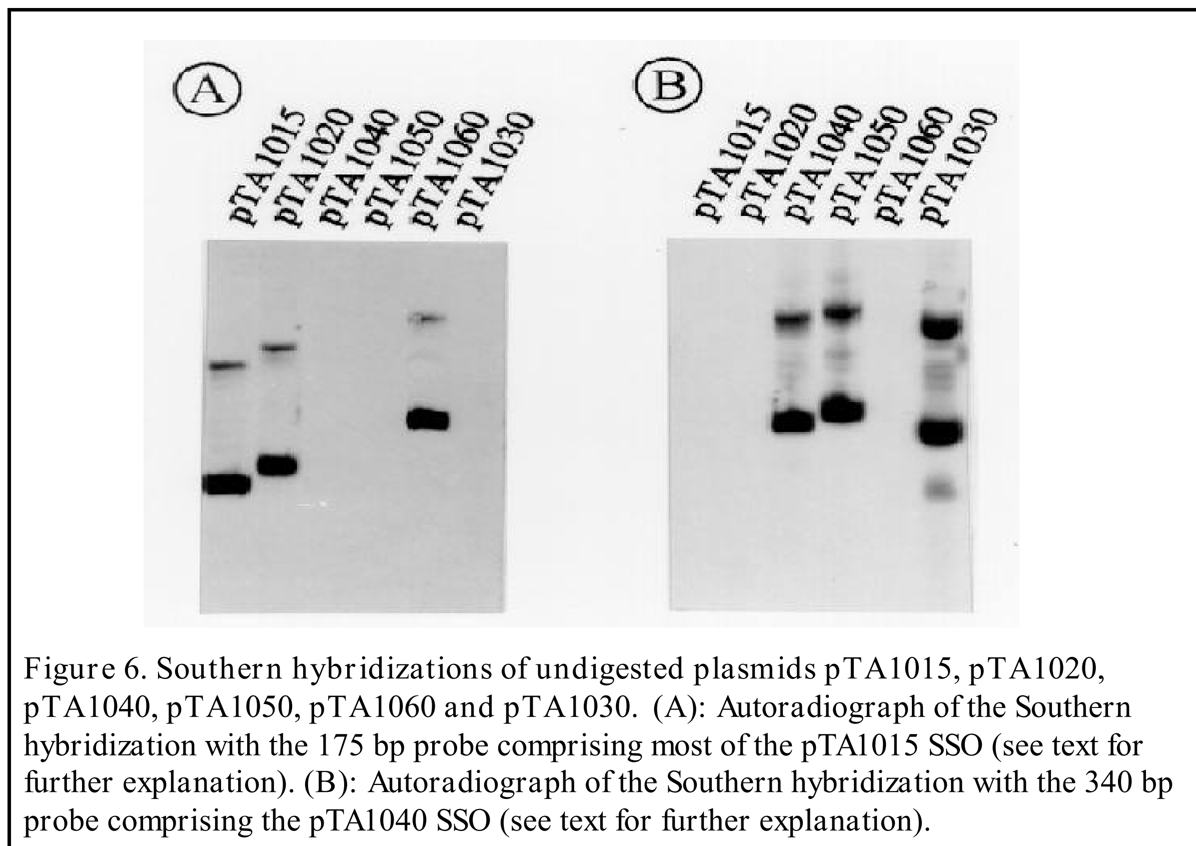

Figure 6. Southern hybridizations of undigested plasmids pTA1015, pTA1020, pTA1040, pTA1050, pTA1060 and pTA1030. (A): Autoradiograph of the Southern hybridization with the 175 bp probe comprising most of the pTA1015 SSO (see text for further explanation). (B): Autoradiograph of the Southern hybridization with the 340 bp probe comprising the pTA1040 SSO (see text for further explanation).

pTA1015. Although not dicussed by the authors (Hara et al., 1992), this region probably functions as the SSO of pUH1. These homologies are also shown in Figure 5. The region between bp 75 and 274 is highly conserved between all five plasmids (at least 96% identity). Outside this region the sequences of pTA1015, pBAA1 and pUH1 are also conserved but these sequences differ from the corresponding sequences in pLS11 and pTA1060. From pLS11 the DNA sequence following bp 242 is not available.

The palT-type family of SSOs can b e divided in two subfamilies

From the foregoing section it is clear that most SSOs from RCM plasmids from B.subtilis described so far, are nearly identical. This raises the question whether palT is the common SSO present on cryptic RCM plasmids from B.subtilis. To answer this question we analyzed the following cryptic plasmids from B.subtilis: pTA1020 of strain IAM1076; pTA1040 of strain IAM1232; pTA1050 of strain IAM1261; pTA1060 of strain IFO3022, and pTA1030 of strain IAM1113. These plasmids have been described by Uozumi et al. (1980). Total DNA extracts were prepared from logarithmically growing cells of each of these B.subtilis strains. After agarose gel electrophoresis and subsequent Southern hybridizations with the respective plasmids as probes, trace amounts of ss plasmid DNA were detected in all cases (results not shown), indicating that each of the plasmids chosen for study uses the RCM of replication.

Next, we studied whether these plasmids contain sequences homologous to palT. For this purpose a 175 bp conserved region of palT (coordinates 75-244, Figure 5) was amplified in a PCR reaction using primers 1 and 2 (see Materials and Methods) and pTA1015 DNA as template.

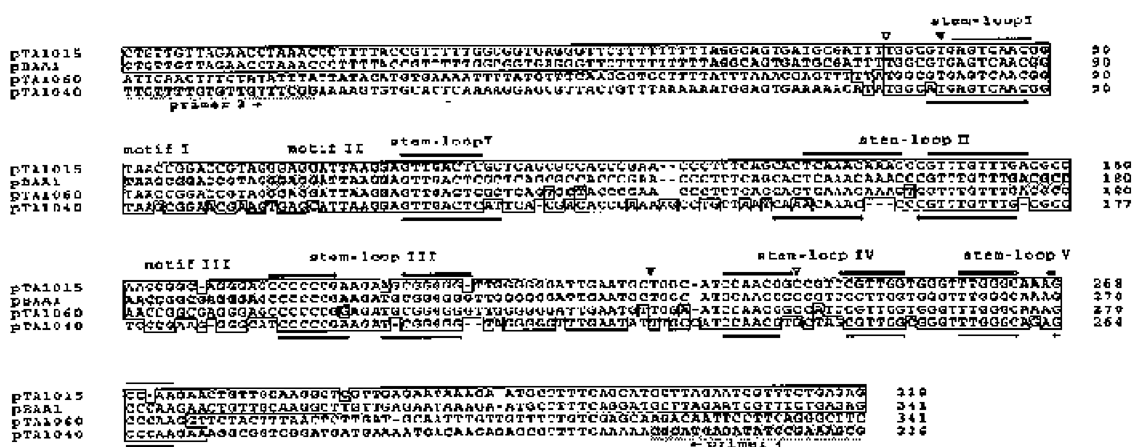

Figure 7. Alignment of the plasmid regions comprising the SSOs of the plasmids pTA1015, pBAA1, pTA1060 and pTA1040. Dyad symmetries are indicated with arrows. The experimentally determined minimal regions required for SSO activity are indicated with

The PCR product was used as a probe for hybridization under stringent conditions to the above-mentioned plasmids. In addition to pTA1015 (positive control), pTA1020 and pTA1060 gave strong hybridization signals (Figure 6A; Figure 6B is discussed later). Based on the high sequence identity (Figure 5) this was to be expected for pTA1060. These results indicate that pTA1020 also contains sequences highly homologous to the palT type SSO. After long exposure times, weak hybridization signals were also obtained with the other three plasmids tested (results not shown). Of these, plasmid pTA1040 was chosen for sequence analysis. On the weakly hybridizing 1.2 kb EcoRI/SphI fragment of pTA1040 a region of 200 bp was identified which showed 77% identity to the palT sequence of pTA1015 (Figure 7). We tested whether this region is a functional ssDNA conversion signal in B.subtilis. For this purpose, a 336 bp fragment, encompassing the region of homology, was amplified by PCR (primers 3 and 4, see Materials and Methods) and cloned in both orientations in the BamHI site of pWM100 resulting in plasmids pWM140 and pWM141 (containing the fragment in the expected functional and non-functional orientations, respectively). The integrities of the cloned fragments were confirmed by sequence analysis. Total DNA extracts from logarithmically growing B.subtilis BD630M15 cells harbouring pWM140 or pWM141 were tested for the presence of ss plasmid DNA. The results (Figure 4) showed that, in contrast to pWM141, no ssDNA was detected with pWM140. This indicates that the inserted fragment of pTA1040 contains a functional SSO which, like other SSOs, is active in only one orientation. Only trace amounts of ssDNA were detected after long exposure times with pWM140 and pWM115 extracts. Similar results were obtained with the native plasmids pTA1015 and pTA1040. These results show that both types of SSO are highly efficient ssDNA conversion signals in B.subtilis. Possible subtle differences in ssDNA conversion efficiency could not be detected by the method used.

Next, we analyzed whether pTA1030 and pTA1050 contain DNA

sequences homologous to the SSO of pTA1040. For this aim, the PCR product described above, which encompasses the SSO of pTA1040, was used as a probe and hybridized under stringent conditions to a blot containing the six pTA plasmids analyzed here. The results (Figure 6B) revealed that, in addition to pTA1040 (positive control), pTA1030 and pTA1050 also gave strong hybridization signals. These results indicate that these latter two plasmids contain sequences perhaps identical, but at least highly homologous to the SSO of pTA1040.

DISCUSSION

In the present work we selected and analyzed SSOs of six RCM plasmids from B.subtilis, which were described by Uozumi et al. (1980). Using the specially designed selection vector, pWM100, the SSO of pTA1015 was cloned by a shotgun approach. SSOs from the other five RCM plasmids were identified by Southern hybridization analysis. The two classes of SSOs described here were highly efficient in the conversion of ssDNA replication intermediates to double stranded plasmid molecules, both when present in their native plasmid or in the correct orientation in pWM100 (SSOs of pTA1060, pTA1015 and pTA1040 were tested).

Sequence analysis of the pTA1015 SSO revealed that it is almost identical to the palT type SSOs which were already known from pTA1060 (our own unpublished results), pLS11 (Chang et al., 1987) and pBAA1 (Devine et al., 1989). Although not mentioned by the authors (Hara et al., 1992), pUH1 also contains this type of SSO. The SSO of pTA1040, which turned out to be 77% identical to the palT of pTA1015, was initially identified by Southern hybridization. pTA1050 and pTA1030 were shown to be highly homologous to the SSO of pTA1040. These findings indicate that in the B.subtilis RCM plasmids described here, two related types of SSOs are present, which we will denote as palT1 (on pTA1015, pTA1020 and pTA1060), and palT2 (on pTA1040, pTA1030 and pTA1050).

We consider it likely that, in additon to the six plasmids analyzed here, nearly all B.subtilis RCM plasmids described so far carry SSOs of the palT1 or palT2 type. This consideration is based on a comparison of published restriction maps and plasmid sizes. Data are available for a series of pTA-plasmids described by Uozumi et al. (1980), a series of pLS-plasmids described by Tanaka and coworkers (Tanaka and Koshikawa, 1977; Tanaka et al., 1977) and a series of pUH-plasmids from Hara et al. (1983). The published physical data allow a classification into six groups of plasmids (Table 1). Plasmids within the same group have identical sizes and restriction profiles and are therefore likely to be identical. Additional support for this view was derived from (i) hybridization studies carried out within the pTA1015 group by Hara et al. (1983) and (ii) detailed restriction profile comparisons between pTA1020 and published data from pBAA1 (our own results, not shown). Each of the six plasmid groups is represented by one of the plasmids studied in the present work. If correct, this classification of B.subtilis RCM plasmids would imply that all plasmids present in the groups represented by pTA1015, pTA1020 and pTA1060, or in the groups represented by pTA1040, pTA1030 and pTA1050, contain the palT1 and palT2 type SSO, respectively.

Table 1. Classification of various cryptic RCM plasmids from B.subtilis

family	type of SSO [A]	size [B]	plasmids with identical sizes and restriction profiles
pTA1015	1	5.8	pTA1015, pTA1010 (=pLS15), pTA1011 (=pLS17), pTA1012, pTA1013 (=pLS19), pTA1014, pTA1016, pTA1017 (=pLS24), pTA1018 (=pLS26), pTA1019, pUH1 through pUH8
pTA1020	1	6.6	pTA1020, pTA1021 (=pLS28), pTA1022 (pLS30), pTA1023, pBAA1
pTA1060	1	8.7	pTA1060 (=pLS11), pTA1061 (=pLS12)
pTA1040	2	7.7	pTA1040 (=pLS13)
pTA1030	2	7.2	pTA1030, pTA1031
pTA1050	2	8.2	pTA1050 (=pLS14)

A: 1 and 2 represent palT1 and palT2 type SSOs, respectively. B: plasmid size in kb. pTA-series according to Uozumi et al. (1980); pLS-series according to Tanaka and coworkers (Tanaka and Koshikawa, 1977; Tanaka et al., 1977); pUH1 series according to Hara et al. (1983).

Detailed studies on the minimal fragment required for a functional SSO have been performed with the plasmids pLS11 (Chang et al., 1987) and pBAA1 (Seery and Devine, 1993). Whereas Chang et al. (1987) focused on the region required for stable plasmid maintenance, Seery et al. (1993) examined the minimal region required for efficient ssDNA conversion. The regions required for the two types of activity (indicated in Figure 7) were nearly identical. The alignments of the palT SSOs of pTA1015, pBAA1, pTA1060 and pTA1040 show that the left border site of the experimentally determined minimal region required for activity coincides with the left starting point of the 200 bp region which is highly conserved between these plasmids. On the other side, sequence conservation continues for approximately 30 bp downstream of the experimentally determined minimal region required for full activity (Chang et al., 1987; Seery and Devine, 1993). Although this indicates that this region is not essential for SSO activity, it might increase the efficiency of the SSO.

SSOs are characterized by their high potential to form secondary structures. For pLS11 (Chang et al., 1987) and pBAA1 (Seery and Devine, 1993) three inverted repeat structures were identified in these SSO regions. Nearly identical structures are also present in the palT2 of pTA1040 (Figure 7). Seery and Devine (1993) have shown that both the sequence and the structure of stem-loop I are critical for SSO activity. Only the potentional stem-loops II and III and their relative position to stem-loop I appeared to be important. In this respect it may be relevant that the differences between the palT2 of pTA1040 and the palT1 sequences hardly affect the stem-loops II and III nor their position relative to stem-loop I.

Within the non-essential 3' part of the conserved region of the four plasmids, conserved fourth and fifth stem-loop structures are present (indicated in Figure 7B). These potential stem-loop structures have not been noted before.

The calculated maximal free energy of stem-loop IV is 13.8 and 9.8 KCal/mol for palT1 and palT2, respectively. For stem-loop V these values are 10.4 Kcal/mol for

both, palT1 and palT2.

Seery et al. (1993) identified three motifs (marked in Figure 7) that were highly conserved between the palT SSO of pBAA1 and the putative SSO of plasmid pGI2, a plasmid isolated from Bacillus thuringiensis (Mahillon and Seurinck, 1988). Recently, homology between palT of pBAA1 and the SSO of another endogenous plasmid from B.thuringiensis, pTX14-3, has been noted (Madsen et al., 1993). In this SSO, the three motifs are also highly conserved. Within motifs 1 and 2, only two basepairs differ between palT of pBAA1 and the corresponding sequences of pGI2 and pTX14-3. Interestingly, these basepairs are conserved between palT2 of pTA10140 and pGI2 and pTX14-3 (bp 94 and 109 in Figure 7A).

In conclusion, our results indicate that most of the identified RCM plasmids from B.subtilis can be placed in one of the six groups defined by Uozumi et al. (1980). SSOs present on these plasmids belong to one of two related subfamilies: palT1 (pTA1015, pTA1020, pTA1060) or palT2 (pTA1040, pTA130, pTA1050). These SSOs are 200 bp in size and the most conserved sequences between the two palT SSO subfamilies coincide with sequences essential for SSO activity. These SSOs are very efficient in B.subtilis, even in heterologous RCM replicons, which implies that they are useful for the construction of stable cloning vectors for this organism.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Bacterial strains and plasmids used are listed in Table 2. TY medium, used for culturing E.coli and B.subtilis, contained Bacto Tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). TY agar contained in addition 2% agar. Erythromycin was added to final concentrations of 2 and 150 µg/ml, and 5-bromo-4-chloro-indolyl-ß-D-galactoside (XGal) to 80 and 40 µg/ml, for B.subtilis and E.coli, respectively.

DNA techniques

All DNA manipulations were carried out according to Sambrook et al. (1989). Restriction enzymes were obtained commercially and used as indicated by the suppliers. Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (Ish-Horowicz and Burke, 1981). Total DNA lysates were prepared as described before (Bron et al., 1988). DNA fragments were isolated from gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, USA). Agarose gels to be used for Southern hybridization 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). Gels were blotted to GeneScreen Plus filters (Dupont NEN, Boston, Ma) using 1.5 M NaCl/0.15 M tri-sodium citrate as transfer buffer. Probe labelling, DNA hybridization conditions and washing steps were performed using the enhanced chemiluminescence DNA labelling 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; Chang and Cohen, 1979). $CaCl_2$-treated E.coli cells were transformed as described by Sambrook et al. (1989).

Table 2. Bacterial strains and plasmids

Strains		Relevant properties	Reference
B.subtilis			
	BD630	hisA1, leu-8, metB5	Albano et al., 1989
	BD630M15	hisA1, leu-8, metB5, lacZ△M15, Km ^{R}	This study
	6GM15	trpC2, tyr1, met, his, ura, rib, r^-_M m^+_M, lacZ△M15, Km ^{R}	Haima et al., 1990
E.coli			
	JM83	F- ara △(lac-proAB) rpsL (Str ^{r}) (Φ80d△(lacZ)M15)	Vieira and Messing, 1982

Plasmids	Properties	Reference
pMV(U⁻A⁻)Em	Broad-host-range, pMV158 derivative lacking palA and palU, Em ^{R}	Meijer et al., 1995b
pUC9	High copy E.coli vector	Vieira and Messing, 1982
pGKH2	pWV01 derivative, Em ^{R}, cat86::LacZ α	Haima et al., 1990
pWM100	pMV158 derivative, Em ^{R}, cat86::LacZ α, no SSOs	This study
pWM160	pWM100 with SSO of pTA1060 in functional orientation	This study
pWM161	pWM100 with SSO of pTA1060 in non-functional orientation	This study
pWM115A,B	pWM100 with SSO of pTA1015 in functional orientation	This study
pWM140	pWM100 with SSO of pTA1040 in functional orientation	This study
pWM141	pWM100 with SSO of pTA1040 in non-functional orientation	This study
pTA1015	5.8 kb, cryptic from B.subtilis IAM1028	Uozumi et al., 1980
pTA1020	6.6 kb, cryptic from B.subtilis IAM1076	Uozumi et al., 1980
pTA1040	7.7 kb, cryptic from B.subtilis IAM1232	Uozumi et al., 1980
pTA1050	8.2 kb, cryptic from B.subtilis IAM1261	Uozumi et al., 1980
pTA1060	8.7 kb, cryptic from B.subtilis IFO3022	Uozumi et al., 1980
pTA1030	7.2 kb, cryptic from B.subtilis IAM1075	Uozumi et al., 1980

Assay of segregational plasmid stability
Single colonies of plasmid-carrying strains were used to inoculate 10 ml of TY medium containing appropriate antibiotics. After overnight growth at 37°C, the cultures were diluted 10^5-fold in prewarmed TY medium without antibiotics (time zero). Samples were taken at appropriate intervals and plated on non-selective TY agar to estimate the total number of viable cells and the number of generations of growth. The fractions of plasmid-containing cells were determined by tooth-picking at least 100 individual colonies onto antibiotic-containing TY agar. After approximately every 10 generations of growth, the cultures

were diluted into prewarmed, non-selective medium to maintain logarithmic growth.

PCR techniques

PCR was carried out essentially as described by Innis and Gelfand (1980). The proofreading-proficient Vent DNA polymerase (New England Biolabs, Beverly, USA) was used throughout. Template DNAs were denatured for 1 min at 94 °C. Next, primers were used to amplify DNA fragments in 30 cycles of denaturation (30 sec; 94°C), primer annealing (1 min; 50°C), and DNA synthesis (3 min; 73°C). Primers used were: 1: 5'-TGGCGTGAGTCAACG; 2: 5'-GGCCGTTGGATGCCA; 3: 5'-GATGGATCCTTGTTTGTGTTGTTTCGG; 4: 5'-GATGGATCCGCTTTCGCATATCTCATGCC. The underlined nucleotides in primers 3 and 4 are extensions to generate BamHI sites.

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