Replication and maintenance of plasmids in Bacillus subtilis

Meijer, Wilhelmus Johannes Jozef

Chapter VIII

Characterization of the replication region of the Bacillus subtilis plasmid pLS20: a novel type of replicon

Wilfried J.J. Meijer, Arjo L. de Boer, Sandra van Tongeren, Gerard Venema and Sierd Bron

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SUMMARY

A 3.1 kb fragment of the large (~ 55 kb) Bacillus subtilis plasmid pLS20 containing all the information for autonomous replication was cloned and sequenced. In contrast to the parental plasmid, derived minireplicons were unstably maintained. Using deletion analysis, the fragment essential and sufficient for replication was delineated to 1.1 kb. This 1.1 kb fragment is located in between two divergently transcribed genes, denoted orfA and orfB, both of which are not required for replication. OrfA shows homology to the B.subtilis chromosomal genes rapA (= spoOL; gsiA) and rapB (= spoOP). The 1.1 kb fragment, which is characterized by the presence of several regions of dyad symmetry, contains no Open Reading Frames (ORFs) of more than 85 codons, and shows no similarity with other known plasmid replicons. The structural organization of the pLS20 minimal replicon is entirely different from that of typical rolling-circle plasmids from Gram-positive bacteria. The pLS20 minireplicons replicate in polA5 and recA4 B.subtilis strains. Taken together, these results strongly suggest that pLS20 belongs to a new class of theta replicons.

INTRODUCTION

The relatively few plasmids that have been isolated so far from Bacillus subtilis strains are all cryptic. Therefore, most vectors used for B.subtilis are based on plasmids containing antibiotic resistance markers which were originally isolated from other Gram-positive bacteria, such as staphylococci and streptococci. Consequently, most information on plasmids replicating in B.subtilis is based on these non-native plasmids (for recent reviews see (Gruss and Ehrlich, 1989; Novick, 1989; Jannière et al., 1993). Based on their mode of replication, plasmids can be divided in two groups: the first group replicates according to the rolling-circle mechanism (RCM) and the second according to the theta mechanism. Most of the small plasmids (smaller than ~ 12 kb) from Gram-positive bacteria use the RCM; the larger plasmids use the theta mechanism. A major distinction between the two modes of replication is the generation of single-stranded (ss) DNA intermediates by RCM plasmids. Several plasmids that use the RCM of replication, like pT181, pUB110, pC194 and pLS1, have been used for vector development. Although such vectors have been used successfully for cloning in B.subtilis, considerable evidence has been provided that the generation of ssDNA replication intermediates is frequently associated with plasmid instability (Ehrlich et al., 1986; Gruss et al., 1987; Peeters et al., 1988; Bron, 1990; Bron et al., 1991; Meijer et al., 1995b; Meijer et al., 1995a).

Apart from the fundamental interest in the replication mechanism of theta plasmids from Gram-positive bacteria, the expectation that these plasmids, which do not generate ssDNA replication intermediates, are structurally more stable than RCM plasmids, has increased the interest for these plasmids. The currently known prokaryotic theta plasmids can be classified in four groups (Bruand et al., 1993). Two of these groups incorporate

plasmids from Gram-positive bacteria that have been studied in considerable detail. One class concerns the broad-host-range streptococcal plasmid pAMß1 (Bruand et al., 1991; Bruand et al., 1993; Jannière et al., 1993; Le Chatelier et al., 1994) and the highly related streptococcal plasmids pIP501 (Brantl and Behnke, 1992; Le Chatelier et al., 1993; Brantl et al., 1994; Brantl, 1994) and pSM19035 (Ceglowski et al., 1993; Rojo and Alonso, 1994), which are all able to replicate in B.subtilis. The other class incorporates a group of highly related narrow-host range plasmids, represented by pWV02 (Kiewiet et al., 1993a), isolated from various lactococcal strains (Seegers et al., 1994). Although it is known that some industrial strains from B.subtilis harbour large plasmids (Le Hégarat and Anagnostopoulos, 1977; Tanaka and Koshikawa, 1977; Bernhard et al., 1978), which probably use the theta mechanism of replication, none of these has been analyzed in detail.

To extend our knowledge on the replication mechanism of theta-replicating plasmids from B.subtilis we analyzed the replication region of plasmid pLS20. Based on its size, approximately 55 kb, we anticipated that pLS20 uses the theta mechanism of replication. Two additional considerations prompted us to carry out these studies. First, we reasoned that endogenous plasmids will be optimally adapted to their host and, therefore, vectors based on replicons of such plasmids might be developed into stable cloning vehicles for B.subtilis, especially for large and heterologous inserts. Second, since B.subtilis is classified as a Generally Regarded As Safe (GRAS) organism, vectors based on endogenous plasmids from B.subtilis are good candidates for the development of food-grade cloning systems in this organism.

pLS20 was originally identified together with an RCM plasmid, pLS19, in the B.subtilis strain IFO3335 (var. natto) (Tanaka and Koshikawa, 1977). Koehler and Thorne (1987) isolated a strain, UM4, which contained only pLS20. We isolated a 3.1 kb region of pLS20 which contains all the information required for autonomous replication in B.subtilis. This fragment was sequenced and the origin region was delineated. The data obtained indicate that, with respect to its mode of replication, pLS20 can not be classified in one of the known groups of plasmids (Bruand et al., 1993) and should be considered as a novel type of replicon.

RESULTS

Construction of plasmids.

Two E.coli plasmids, pTC1 and pKM2 (Fig. 1), were constructed for these studies. pTC1 is a derivative of the low-copy number, pSC101-based, replicon pHSG575 (Takeshita et al., 1987) which contains the multiple cloning site of pUC8. The tetracycline resistance (Tc^R) gene of pMV158, which is expressed in Gram-positive as well as in Gram-negative bacteria, was introduced into pHSG575. For this purpose, a unique BamHI site was first introduced using PCR techniques into pMV158 upstream of the promoter of the Tc^R gene [position 1555 according to (Lacks et al., 1986)]. Next the 1.6 kb BamHI/EcoRI fragment of pMV158 containing the Tc^R gene was cloned into the corresponding sites of pHSG575, resulting in pTC1.

pKM2 (Fig. 1B) is a derivative of the high-copy number E.coli plasmid pMTL25 which contains an extended symmetrical multiple cloning site and several unique sites (Chambers et al., 1988), into which the kanamycin resistance gene (Km^R) from the Streptococcus faecalis plasmid pJH1 was cloned. For this purpose, the 1.3 kb HindII fragment of pKM1 (Kiel et al., 1987), containing the Km^R gene, was

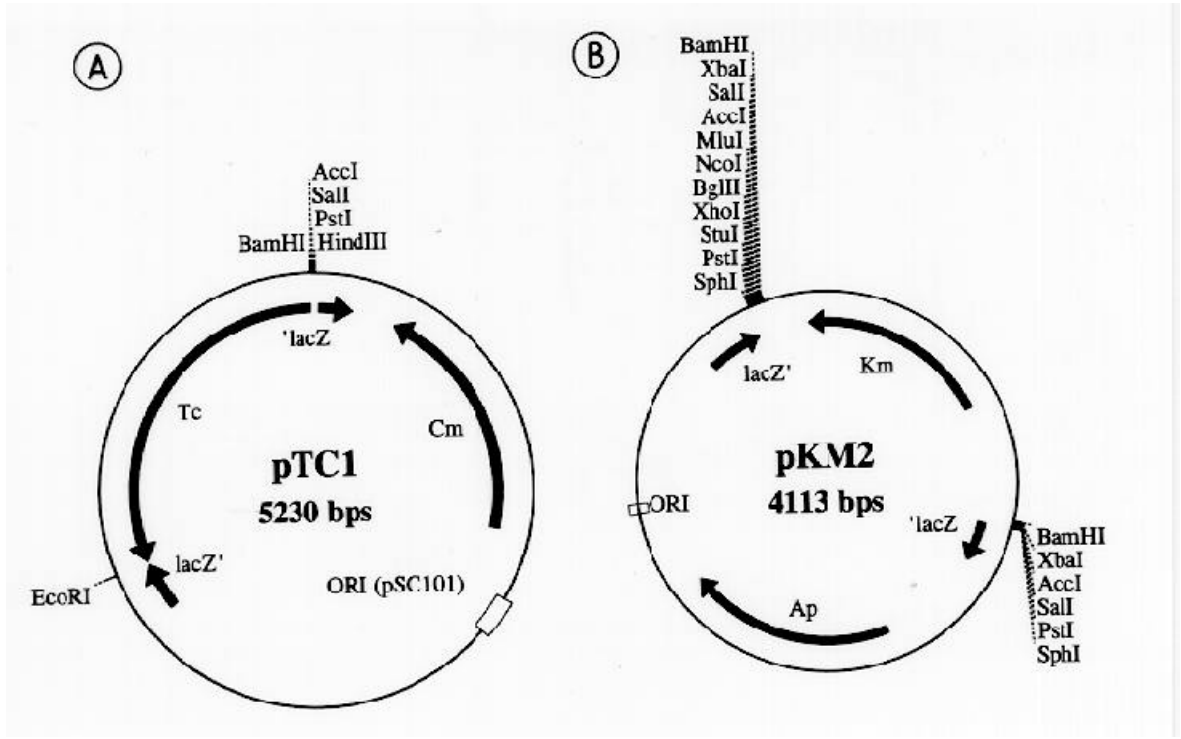

Figure 1. Schematic representation of the E.coli vectors used.
(A) The low-copy number vector pTC1 based on the replication functions of pSC101; and (B) the high-copy number plasmid pKM2 based on pMTL25. Relevant restriction sites are indicated; lacZ' and 'lacZ indicate the 5' and 3' regions of the lacZ " gene; ORI, origin for replication in E.coli; Tc, tetracycline

cloned into the SmaI sites of pMTL25, resulting in pKM2. Additional advantages of this construction were that the multiple cloning site of pMTL25 still contained unique sites for the cloning of additional DNA fragments and that the plasmid regions required for replication in E.coli could easily be deleted from the resulting clones due to the presence of symmetrical sites in the multiple cloning site.

Derivatives of pTLS6 were contructed in E.coli by deleting the following, pLS20-derived, fragments (Fig. 2): the 5.0 kb BamHI/SmaI fragment resulting in pTLS6Δ5; the 2.2 kb BamHI/SphI fragment resulting in pTLS6Δ2; and the 3.9 kb SalI/Eco47^{III} fragment resulting in pTLS6Δ4. Plasmid pEBS4 was constructed by cloning the 4 kb BamHI/SmaI fragment of pTLS6 in a pBluescript vector containing the erythromycin resistance marker of the S.aureus plasmid pE194 [pEBSK, (Kiewiet et al., 1993a)].

Cloning of the minimal replicon of pLS20.

In order to clone the replication region of pLS20, the plasmid was partially digested with Sau3A. After agarose gelelectrophoresis the fragments ranging from about 1 to 15 kb were isolated and ligated to BamHI-linearized pTC1. The pSC101-based pTC1 plasmid is unable to replicate in B.subtilis. The ligation mixture was used to transform B.subtilis PSL1 protoplasts. The few Tc^R transformants obtained all contained an identical insert of approximately 12 kb, indicating that this fragment contained the infomation required for replication in B.subtilis. One of the

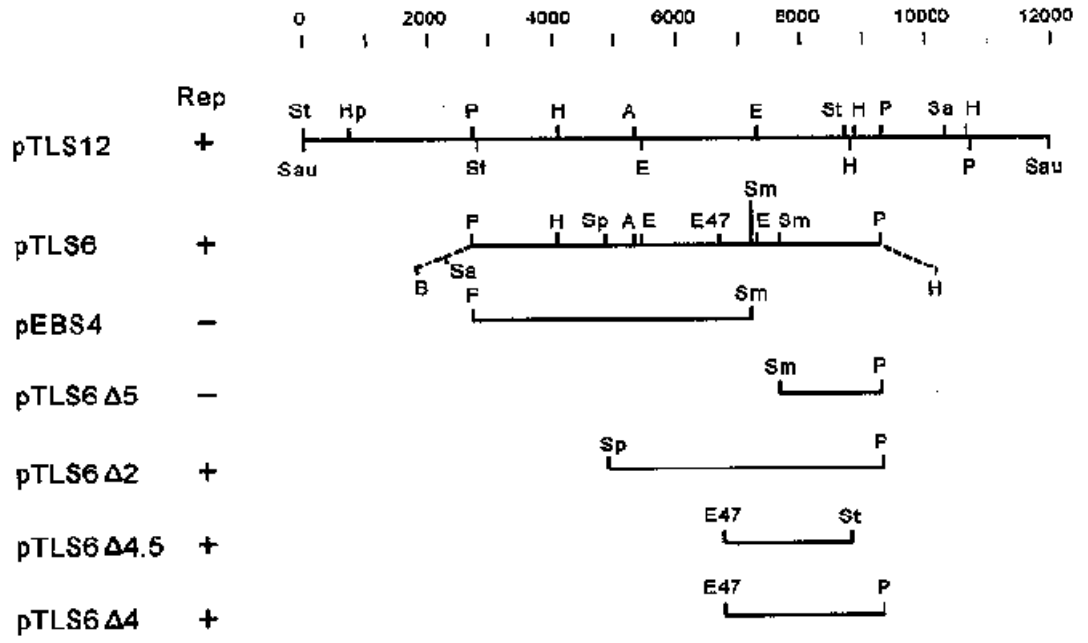

Figure 2. Schematic representation of the 12 kb region of pLS20 containing the replication functions and several deletion derivatives.
(+) and (-) indicate the ability or inability to replicate in B.subtilis. The restriction sites at the borders of pTLS6 (shown as interrupted lines; B and Sa on the left and H on the right) represent sites originating from the multiple cloning site of the pTC1 vector. Only relevant restriction sites are indicated. Sau, Sau3A;

recombinant plasmids, designated pTLS12, was used for further analysis. A restriction map of the 12 kb insert is shown in Fig. 2. The replication region was further delineated by subcloning the PstI fragments of the 12 kb insert into the unique PstI site of pTC1. Only plasmids containing the largest PstI fragment of approximately 6.5 kb were able to replicate in B.subtilis. One of these, denoted pTLS6 (Fig. 2), was taken for further analysis. To verify that the cloned fragments of 12 and 6.5 kb in pTLS12 and pTLS6 originated from pLS20, these inserts were hybridized under stringent conditions to EcoRI digests of purified pLS20, total DNA extracts of the B.subtilis strain UM4 (pLS20), and the plasmid-free B.subtilis strains PSL1 and 8G5. As expected, a hybridization signal was only obtained with pLS20 and UM4-extracted total DNA (results not shown). To further delineate the pLS20 replication region, the pTLS6 deletion derivatives pTLS6Δ5, pTLS6Δ2 and pTLS6Δ4, and plasmid pEBS4 were constructed in E.coli (Fig. 2). When these plasmids were used to transform B.subtilis cells, transformants were only obtained with pTLS6Δ2 and pTLS6Δ4. These results showed that all information required for replication was present on the 2.9 kb PstI/Eco47^{III} fragment of pLS20. In addition, from the observation that pTLS6Δ5 can not replicate in B.subtilis, it is clear that an essential region for replication is located on the 0.95 kb Eco47^{III}/SmaI fragment which is present in pTLS6Δ4, but absent in pTLS6Δ5. pTLS6Δ4.5, also shown in Fig. 2, was constructed after the sequence of the 2.9 kb Eco47^{III}/PstI fragment had been determined and this plasmid will be discussed in a subsequent section.

Sequence of the 2.9 kb PstI/Eco47III fragment containing the pLS20 replication functions.

The 2.9 kb PstI/Eco47III fragment of pLS20, as present in pTLS6Δ4 containing the replication functions, was sequenced double-stranded from overlapping subclones in pUC18. The results are shown in Fig. 3. The nucleotide sequence has been deposited in the EMBL/Genbank/DDBJ nucleotide sequence database and was assigned the accession number U26059. Evidence that this 2.9 kb DNA region is a contiguous fragment of pLS20 was obtained from PCR reactions using the plasmids pTLS6Δ4 and pLS20 as templates and various sets of primers derived from the DNA sequence. With both plasmid templates the sizes of the PCR products were as expected from the sequence of the 2.9 kb fragment (results not shown). Analysis of this sequence revealed the presence of two open reading frames (ORFs) encoding putative proteins of more than 100 amino acids, each containing a putative start codon preceded by a potential ribosomal binding site (RBS). These ORFs, designated orfA and orfB, are divergently transcribed relative to each other. Several properties of these orfs are summarized in Table 1. The frame of orfB is still open at the Eco47^{III} site which defines the end of the cloned fragment. Therefore, in the sequence shown, orfB is truncated after the 110th codon. The deduced orfA product has 45% similarity with two chromosomally-encoded B.subtilis protein-aspartate phosphatases: rapA [originally identified as a glucose-starvation-induced gene (gsiA; (Mueller et al., 1992), which was also named spoOL] and rapB [formerly named spoOP (Perego et al., 1994)]. Rap stands for response regulator aspartate phosphatase (Perego et al., 1994). The chromosomal rapA gene (called gsiAA by Mueller et al. (1992)) is followed by a translationally coupled small gene, gsiAB, specifying 44 amino acids. Also, orfA of pLS20 is characterized by the presence of a translationally coupled ORF, which we will designate as orfAB. Due to the cloning procedure, orfAB was truncated after the 21st codon in pTLS6Δ4. The DNA sequence of the complete orfAB was determined, however, by sequencing 250 bp downstream of the PstI site using pTLS12 as template (bp 2881 to 3136 in Fig. 3). The additional sequence data showed that, like gsiAB, also orfAB specified a putative protein of 44 amino acids (Table 1). Directly downstream of orfAB an inverted repeat structure is present which is likely to function as a rho-independent transcriptional terminator (Fig. 3). In all these aspects, the structural organization of the pLS20 orfA and orfAB genes resembles that of the chromosomal rapA genes. Contrary to orfA, no significant homology was observed between the truncated orfB and available sequences in databanks.

The deduced products of orfA, orfAB and orfB do not share homology with known replication proteins. The intergenic region between orfA and orfB contains a number of small ORFs (<85 codons, Fig. 3). However, these do not contain a potential RBS at appropriate distances from potential start codons, nor do the deduced amino acid sequences show homology to known proteins. Thus, the region sufficient for autonomous replication does not seem to encode a replication initiation protein. Although it is most unlikely that a replication initiator protein is located elsewhere on pLS20 and that a functional equivalent would be provided by the B.subtilis chromosome, we cannot totally exclude this possibility.

Table 1. Properties orfA, orfB and orfAB genes

Genes	Position [a]	Size [c]	Mol. mass (kDa) [d]	Putative startcodon and RBS [e]	Spacing (bp) [f]
orfA	1720 - 2823	368	43.4	ttgaatagct ggagg gaagtgtATG	9
orfAB	2823 - 2954	44	4.6	aaagaataac ggagg cgttaggATG	9
orfB	332 - 1 (C) [b]	> 110	- -	aatc aaaggag agaataaaaatATG	12

a: The numbers correspond to positions in Fig. 3
b: "C" indicates that the ORF is located on the opposite strand
c: Size in codons
d: Molecular masses are calculated from the deduced amino acid sequences
e: The putative initiation codon (capital letters) and the 22 bp upstream region are shown; nucleotides complementary to the 3' end of B.subtilis 16S rRNA UCUUUCCUCCACUAG (Moran et al., 1982) are underlined.
f: The spacing is calculated as the distance from the first base to the right side of the AGGA sequence (or the equivalent) to the base adjacent to the initiation codon.

The following characteristic features were identified in the 1.4 kb intergenic region between orfA and orfB by computer-assisted analysis.

(A) Six inverted repeated sequences are present in this region that have the potential to form stem-loop and hairpin structures. These sequences are marked in Fig. 3 and their positions are indicated in Fig. 5. The maximal calculated free energies of these structures are, respectively: -12.4, -15.4, -11.8, -31.4, -9.2 and -38.2 kCal/mol.

(B) Regions with high and low AT-contents were identified. Whereas the overall AT-content of the 3.1 kb fragment is 63%, the region from bp 890 to 1100 has an AT-content of only 47% (for position see Fig. 5). Several small regions have a high AT-content (> 85%): (i), from position 1463 to 1487 (25 bp with 92% AT); (ii), from position 674 to 709 (35 bp with 86% AT); (iii), from position 442 to 457 (17 bp with 94% AT); and (iv), from position 387 until 407 (19 bp with 85% AT). Third, several (imperfect) direct repeats were identified (Fig. 3). The direct repeats are (i), 5'-AAAATGAAATCA-3' (starting at positions 397 and 453); (ii), 5'-AAATCAGTGAA-3' (starting at positions 414 and 459); and (iii), 5'-GAAATCAGT-3' (starting at positions 458 and 467). These repeats form part of sequences which are also recognized for their high AT-content. Another region recognized for its high AT-content (position 673 to 708) contains three times the nearly identical sequence 5'-ACAAATAAAAAG-3'. (C) Several sequences were identified showing homology to DNA sequences known to be involved in replication. (i) DnaA boxes were identified starting at the following positions (coordinates according to Fig. 3): 1038 (consensus DnaA box [5'-TTATCCACA-3'], lower strand); 998 (2 mismatches compared to consensus; [5'-TTcaCCACA-3'], lower strand); 1029 (3 mismatches compared to consensus; [5'-TTcTCCggA-3'], lower strand); and 960 (3 mismatches compared to consensus; [5'TataCCACA]). All potential DnaA boxes are located within the region of low AT-content. (ii) Upstream of orfA

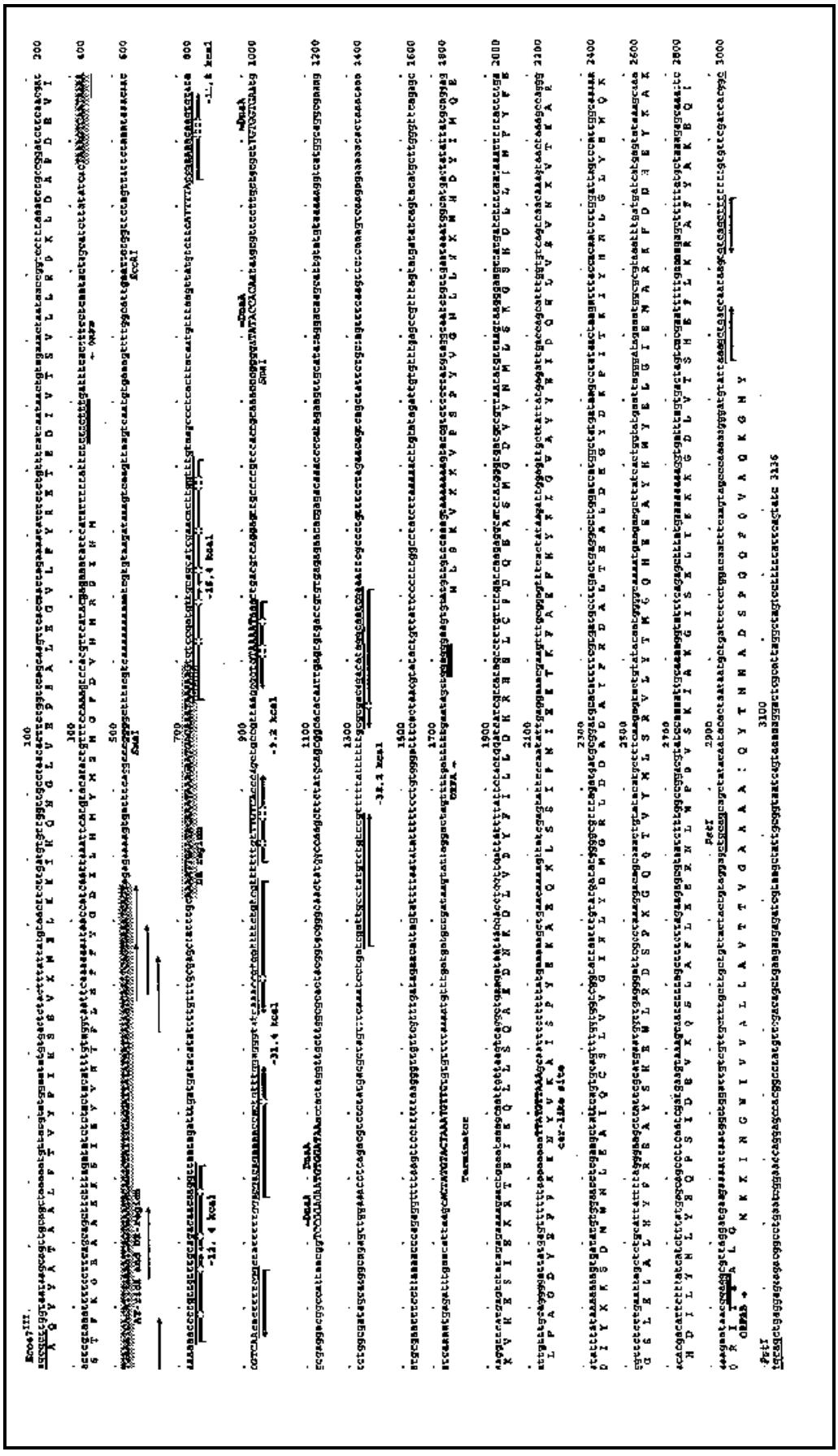

Figure 3. DNA sequence of the 3136 bp fragment of pLS20 containing all the information required for autonomous replication.
The putative amino acid sequences of ORFA, ORFAB and ORFB are presented below the DNA sequence. Possible Shine-Dalgarno sites are double underlined; inverted repeated sequences are indicated with double-line arrows; single-line arrows indicate direct repeats. The following characteristics are indicated: Terminator is a DNA replication terminator; DnaA is consensus DnaA-box; . DnaA is a DnaA-like box; cer is a cer-like resolution site. Regions characterized by a high AT content and including direct or imperfect direct repeats are shaded. The following short ORFs, lacking a potential RBS at an appropriate distance from the putative startcodon, are present in between the divergently transcribed orfA and orfB: (i) lower strand, position 727 to 536: MLQHRTLFIC-HSLFVFNFAN-SSQTKYVSSN-LLTLLSANNR-VFCCLFRITR-TRIQCQNFHI-DLT; (ii) upper strand, position 998 to 1246: MARTAINGPE-NVDKPLGCLA-HYGAGNYGQA-SMQRHTIECD-RVREHETNPI-EVAYRTSIVC-KKVMAGESLA-IARQSWKPQT-SL; (iii) upper strand, position 1243 to 1395: MTLVSNPDRL-PMSVRFYFLR-DRHRQSKFAL-FPRTVSYSAS-FKFSKSREKH; and (iv) upper strand, position 759 to 923 :
MFKLCLHFTE-NKCIRQHFSA-IFGRQKTTVW-RVSNRGFLSF-FVCHPAAVKG-CKIN.

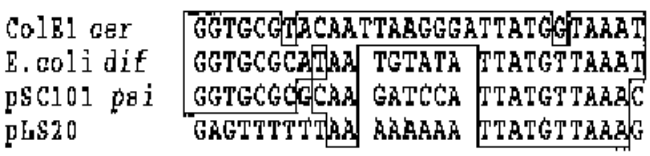

Figure 4. Potential site-specific recombination site of pLS20. Alignment between the RecA-independent recombination sites dif of the E.coli chromosome, cer and psi of the E.coli plasmids ColE1 and pSC101, respectively, and the homologous region of pLS20. Identical sequences are boxed.

(positions 1626 to 1644) a DNA region of 18 bp (5'-CACTATGTACtAAATGTTC-3') was recognized which, except for a 1 bp mismatch, is identical to part of the B.subtilischromosomal DNA terminator (Carrigan et al., 1987). Replication termination activity of a fragment encompassing this region has been demonstrated; the results of these studies will be published elsewhere (Meijer et al.; in preparation).

(iii) A short DNA sequence of 10 bp within the coding sequence of orfA was identified (position 2037 to 2046 in Fig. 3: 5'-TTATGTTAAA-3') which is identical to part of the E.coli chromosomal XerCD recombination site (dif) (Kuempel et al., 1991) and the equivalent psi (Cornet et al., 1994) and cer sites (Summers and Sherratt, 1984) of the E.coli plasmids pSC101 and ColE1, respectively (alignments are shown in Fig. 4).

Delineation of the pLS20 replication region.

As mentioned in a foregoing section, neither the ORFA nor the ORFB product show homology to known replication initiation proteins, suggesting that these gene products are not essential for replication. Support for this idea was the observation that pTLS6Δ4.5 (deletion of the 3'-terminal half of orfA) was still able to replicate in B.subtilis, despite the fact that orfB and orfA are truncated in this construct (Fig. 2). To prove that the putative products of both ORFs are dispensable for replication, and to further delineate the replication region, various PCR-amplified fragments of the intergenic region between orfA and orfB were subcloned in the E.coli vector pKM2. The resulting plasmids were tested for their ability to replicate in B.subtilis. The positions of the primers, listed in

Table 2. Primers used

Name	Position*	Sequence (5'-3')#
LS20-1	345 →	ggggaTCCTTTGATTTCACTTTCCTC
LS20-2	2139 ←	ggggaTCCAATCTTATAGTGAAACTCCGC
LS20-3	491 →	ggggatcCAGGCCCGGGGCTTTACGTC
LS20-4	1722 ←	ggggatcCAACATACACTTCCCTCCAGCT
LS20-5	936 →	ggggatCCCCGTCCACCGCAAACCCGGGG
LS20-6	1592 ←	ggggatCCCAAGCATGTACTGATATC

*: Positions are according to Fig. 3, directions of primers are indicated with arrows.
#: Nucleotides identical to pLS20 sequences are in upper case letters. Lower case letters represent 5' extensions specifying BamHI sites. SmaI sites are shown in bold.

Table 2, are indicated in Fig. 5. Primers 1 and 4 were deliberately chosen to overlap with the potential RBS sequences so that the resulting fragments would include the promoters of orfA and orfB. Primers 3 and 6 were located approximately 150 bp upstream of these RBS sequences. Nine regions were amplified by using pTLS6Δ4 as template DNA and nine different sets of primers (primer 1, 3 or 5 each combined with primer 2, 4 or 6). The primers were extended with a BamHI site (not present in the orfA-orfB intergenic region). The amplified PCR products were digested with BamHI and ligated to BglII-linearized pKM2 DNA. The ligation mixtures were used to transform B.subtilis PSL1 protoplasts. Kanamycin resistant transformants were obtained with six out of the nine ligation mixtures used (only the three amplified regions in which primer 5 was used failed to transform B.subtilis when cloned in pWKM2). Restriction analyses of plasmid DNA isolated from the transformants revealed that all inserts were obtained in both orientations. The smallest region containing all the information enabling autonomous replication in B.subtilis was obtained using primers 3 and 6. The resulting plasmid was designated pWKM6 (Fig. 5). No transformants were obtained with pKM2 vector DNA alone. The possibility that DNA sequences of the pKM2 vector together with pLS20 sequences are required for autonomous replication of the various constructs obtained was excluded by the following experiment. DNA of these constructs was digested with PstI (Fig. 1). The resulting fragments, containing only the Km^R gene and the corresponding pLS20 inserts, were isolated, self-ligated and used to transform competent B.subtilis 8G5 cells. In each case Km^R transformants were obtained which, as judged from restriction analyses, all contained the expected plasmids. The observation that pWKM6 was able to replicate in B.subtilis proved that the ORFA/ORFB products are not required for pLS20 replication in B.subtilis. Although the positions of the promoters of orfA and orfB have not been mapped, it is likely that these are not present in pWKM6, since a region of about 150 bp upstream of the

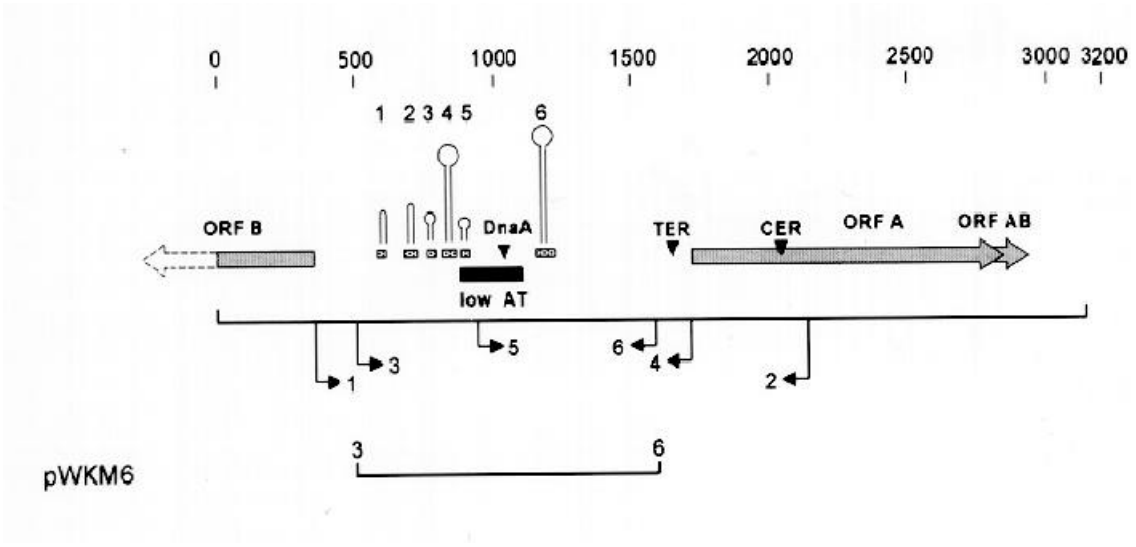

Figure 5. Schematic representation of the pLS20 replication region.
The positions of the primers used for amplification of the various regions of pLS20 are indicated. pWKM6 is a pKM2 derivative containing the indicated pLS20 region. The following characteristics are shown: orfA, orfA B and orfB (arrows); consensus DnaA-box (DnaA); DNA replication terminator (Ter), and cer-like site (cer) (the latter three characteristics are indicated with triangles). The six regions of dyad symmetry are numbered 1 through 6. The 200 bp region with a low AT-content is marked with a filled bar indicated as "low AT". (+) and (-) indicate ability or inability to drive autonomous replication in B.subtilis.

RBSs of these genes are lacking. Therefore, promoter activity of these genes is probably not necessary for pLS20 replication. In addition, this result shows that neither the proposed cer and ter sites, nor the region from 387 to 475 which comprises three direct repeats and two AT-rich regions, were essential for replication. No differences in plasmid copy numbers were observed between the various constructs. As judged from ethidium-bromide stained agarose gels the constructs had a copy number of 2 to 4 per chromosome equivalent (data not shown).

The observation that the three constructs containing the pLS20 region that was obtained when primer 5 was used, were unable to replicate in B.subtilis, in contrast to the situation when primer 3 was used, indicates that a feature essential for pLS20 replication is located on the 0.44 kb SmaI fragment (positions 491 to 936 in Fig. 3). This is in agreement with the observation that pTLS6Δ5, also lacking this SmaI fragment, was not able to replicate in B.subtilis. Characteristics identified in this 0.44 kb SmaI region are five of the six identified regions with dyad symmetry and the AT-rich region containing three times the nearly identical repeat 5'-CTTTTTATTTGT-3.

Properties of the pLS20 minireplicon.

To further characterize pLS20 replication we analyzed: (i), maintenance of miniderivatives; (ii), production of ssDNA replication intermediates; (iii), thermoresistance of replication; (iv), dependence of replication on the host-encoded DNA polymerase I (PolI) enzyme; (v), effects of prevention of translation on plasmid copy numbers.

(i) Maintenance of pLS20 miniderivatives. Although pLS20 has a low plasmid copy number (1 to 3 per chromosome (Koehler and Thorne, 1987)),

it is maintained very stably. Plasmids with low copy numbers generally encode functions to ensure their stable maintenance (Nordström and Austin, 1989; Williams and Thomas, 1992). It was, therefore, of interest to analyse whether such functions were associated with the pLS20 minireplicons described in the foregoing sections. To investigate this, the maintenance of pTLS12 and pTLS6 was compared with that of the parental plasmid pLS20. Whereas pLS20 was maintained fully stable over 80 generations of growth, only about 25% of the cells harboured pTLS12 or pTLS6 under similar conditions (results not shown). This makes it likely that pLS20 contains functions involved in stable plasmid maintenance that are located outside of the 12 kb region present in pTLS12.

(ii) Production of ssDNA replication intermediates. A major distinction between rolling-circle and theta replication is the production of ssDNA replication intermediates in the former system. To study the possibility that pLS20 uses the RCM of replication, we analyzed whether pLS20 miniderivatives produced ssDNA. For this purpose, total DNA extracts were prepared from B.subtilis PSL1 cells harbouring either the pLS20 derivatives pTLS12 or pTLS6, or the RCM plasmid pLS1 which is known to accumulate ssDNA in B.subtilis (Meijer et al., 1995a). After gel electrophoresis and Southern hybridization with appropriate probes, ssDNA was only detected with pLS1 (results not shown). This makes it unlikely that pLS20 uses the RCM of replication.

(iii) Thermoresistance of pLS20 replication. Although the theta-replicating plasmid pAMß1, originally isolated from Streptococcus faecalis, is able to replicate in B.subtilis, its replication is thermosensitive in this bacterium (Jannière et al., 1990). Also replication of the Bacillus thuringiensis theta plasmid pTH1030 was reported to be thermosensitive in B.subtilis (Lereclus et al., 1988). Vectors based on these plasmids can not replicate at 47 °C and, therefore, can not be used in thermophylic Bacillus species. To study whether pLS20 may be a suitable vector for thermophylic Bacillus species, its ability to replicate at 37 and 49 °C was studied. Appropriate dilutions of cultures of B.subtilis harboring pTLS6, pTLS6Δ4, pWKM1, pWKM2 or pWKM3 were plated on TY agar and incubated at either 37 or 49 °C. No significant differences were observed between the number of colony-forming units or the size of the colonies after overnight growth at the two temperatures. Moreover, no temperature effect was observed on the copy number of pWKM1 (results not shown). These results indicate that pLS20 replication is not thermosensitive and that vectors based on the replication functions of pLS20 may be useful for thermophylic Bacillus species.

(iv) Dependence of the host-encoded PolI enzyme. To investigate whether replication of pLS20 depends on functional PolI, plasmids pWKM1 through pWKM6 were tested for their ability to transform the polA5 B.subtilis strain 1A226 and the isogenic PolI-proficient strain 1A224. The polA5 strain 1A226 lacks PolI, which was shown by genetic, biochemical and partial sequence analyses (Mazza and Galizzi, 1978; Mazza and Galizzi, 1989; Bruand et al., 1993). Whereas rolling-circle replicating plasmids, like pUB110, can replicate in the polA5 strain, the theta-replicating derivative pIL252 of plasmid pAMß1 is unable to do so (Bruand et al., 1993). Therefore, these plasmids were used as positive and negative controls for pLS20 derivatives. The results, presented in Table 3, show that all pLS20 derivatives tested transformed both the polA5 strain and the isogenic PolI-proficient strain efficiently to kanamycin resistance. As expected, the control plasmids pUB110 and pIL252 transformed the PolI-proficient strain efficiently to antibiotic resistance, whereas

Table 3. PolI independency for replication of pLS20 derivatives

Plasmid	Number of Km^R transformants obtained	
	1A224 (wt polI)	1A226 (polA5)
pWKM1	$1.6*10^3$	$2.0*10^3$
pWKM2	$6.4*10^3$	$9.4*10^3$
pWKM3	$8.9*10^3$	$1.3*10^4$
pWKM4	$4.4*10^3$	$1.1*10^4$
pWKM5	$9.6*10^3$	$1.7*10^4$
pWKM6	$0.3*10^3$	$0.2*10^3$
pUB110	$2.0*10^4$	$1.4*10^5$
pIL252	$3.9*10^3$	0

only pUB110 transformed also the polA5 strain. For each transformation, eight antibiotic resistant transformants were analyzed for their plasmid content. All of them contained the expected plasmid.

(v) Effects of preventing translation on plasmid replication. As described in one of the foregoing sections, the replication region of pLS20 does not encode a Rep protein and, in this respect, resembles the ColE1-type plasmids of E.coli. We determined whether the copy numbers of pLS20 derivatives were affected by Cm. After the addition of 100 µg/ml Cm to cultures of logarithmically growing cells harbouring either pWKM2 or pWKM3, samples of the cultures were taken as a function of time and the plasmid copy numbers were determined. No increase in copy numbers was observed even at 4 hours after the addition of Cm (results not shown).

DISCUSSION

Two modes of DNA replication are known for bacterial plasmids: the rolling-circle mechanism (RCM) and the theta mechanism. Plasmids replicating according the RCM are easily recognized by (i), their limited size (<12 kb); (ii), the generation of ssDNA replication intermediates; and (iii), the presence of several functional modules. Examples of these modules are a rep gene encoding the essential replication initiator protein, and modules comprizing the initiation sites for leading- and lagging-strand synthesis (for a review see reference (Gruss and Ehrlich, 1989)). None of these typical features of RCM plasmids applied to pLS20: (i) pLS20 is large (~ 55 kb); (ii), no ssDNA intermediates were detected; and (iii), no characteristic modules of RCM plasmids were identified. These data indicate that pLS20 uses the theta mechanism rather than the RCM of replication. In support of the view that pLS20 is a theta-type plasmid, several features were identified which are typical for theta-replicating plasmids. These were the presence of: (i), DnaA boxes; (ii), an AT-rich region containing several imperfect direct repeats; and (iii), a replication terminator (Meijer et al., in preparation). Although these properties are no absolute proof, these data strongly suggest that pLS20 uses the theta mechanism of replication.

The 1.1 kb region, sufficient for pLS20 replication, is characterized by the presence of several inverted repeat sequences and the absence of ORFs larger than 85 codons. Because the small ORFs lack appropriate translational start signals it is unlikely that pLS20 replication requires plasmid-specified proteins. This

raises the intriguing question how replication of pLS20 is initiated. The currently known theta plasmids can be classified in four groups, designated A through D (Bruand et al., 1993). This classification is based on three characteristics: the presence or absence of a Rep protein; the presence of an oriA-like structure; and the dependence of replication on the host-encoded PolI. Class A includes plasmids which encode a replication protein (Rep) and these plasmids have a characteristic replication origin, designated oriA (Kornberg and Baker, 1992). Typically, oriA contains repeated sequences (iterons) recognized by the cognate Rep protein, one or more DnaA boxes, and an AT-rich region which generally contains repeats. PolI is not required for this type or replicon, which is exemplified by the E.coli plasmids R1, pSC101, F, RK2, P1 and R6K. The group of related plasmids isolated from various Gram-positive lactococcal and streptococcal strains, exemplified by pWV02 (Kiewiet et al., 1993b; Seegers et al., 1994), are likely to belong to this class. Class B replicons do not encode a Rep protein and lack the typical oriA region (Kornberg and Baker, 1992). Their replication is initiated by processing of a transcript synthesized by the host RNA-polymerase. The processed transcript is used as a primer for leading strand synthesis, which is initially carried out by PolI and later taken over by the replication enzyme complex (Kornberg and Baker, 1992). ColE1 is the prototype of this family of plasmids. Class C contains a group of at least 17 related plasmids, collectively called ColE2-related plasmids (Hiraga et al., 1994). These plasmids encode a Rep protein essential for replication (Horii and Itoh, 1988; Itoh and Horii, 1989; Kido et al., 1991) and they require the host-encoded PolI (Kingsbury and Helinski, 1970; Tacon and Sherratt, 1976). Recently, the understanding of the underlying replication mechanism for this class of plasmids has been increased considerably by the discovery that the Rep proteins involved bind to their cognate origin, located directly downstream of the rep gene (Kido et al., 1991), and synthesize a unique primer RNA (ppApGpA) which is used for initiation of leading-strand synthesis by PolI (Takechi et al., 1995; Takechi and Itoh, 1995). Like class C plasmids, class D plasmids, represented by the S.faecalis plasmid pAMß1, encode a Rep protein and require PolI for the initiation of replication. Although an oriA-like structure was identified upstream of the rep gene, this structure is not required for replication (Bruand et al., 1993). Since the exact function of the Rep protein is unknown it is possible that the replication mechanism of class D plasmids is analogous to that of the class C plasmids.

pLS20 can not be classified in one of the four known classes of theta replicons. First, unlike plasmids belonging to class A, C and D, pLS20 does not encode a Rep protein. Second, unlike ColE1 (class B plasmids), pLS20-derived replicons could be established in a PolI-mutated strain, and its copy number was not affected by Cm treatment. What than could the mechanism of replication of pLS20 be ? The final answer to this question can presently not be provided, but the presence of specific sequences within the pLS20 replicon that show homology with sequences involved in replication of other replicons suggests that these sequences are involved in replication. Although pLS20 does not encode a Rep protein, it shares features with the class A-type plasmids: (i), its replication is independent of PolI, and (ii) its replication region contains DnaA boxes and an AT-rich region with imperfect direct repeats, elements which are typical for oriA-like structures. The AT-rich regions within oriA structures of class A plasmids are believed to constitute the first regions to become melted during replication. The AT-rich

region identified in pLS20 (position 674 to 709, Fig. 3) is located on the 0.44 SmaI fragment which is essential for pLS20 replication. Possibly, this region of pLS20 functions as the first region to be melted during initiation of replication. An alternative explanation for the requirement of this SmaI region for replication is based on the presence of several inverted repeated sequences with the potential to form stem-loop structures. In several eukaryotic viruses, e.g. Simian virus 40 (SV40), Herpes simplex virus 1 (HSV-1) and Epstein-Barr virus (EBV) the initial melting region is located in a palindromic sequence (Kornberg and Baker, 1992). In these viruses and some bacteriophages the initial melting step is affected by transcriptional activity at or nearby the origin (Kornberg and Baker, 1992). In this respect it is worth mentioning that the IR 4 in the pLS20 minireplicon is flanked by a pair of divergently oriented σ^A- dependent promoter-like sequences (positions 884 to 916: 5'-TTGtCA ··· [21 nt] ··· TAaAAT-3'; and positions 806 to 778: 5'-TTGACg ··· [17 nt] ··· TAaAAT-3'). Conceivably, the palindrome, perhaps in conjunction with transcriptional activity, is required for replication. This idea is partly based on analogies with the replication region of the B.thuringiensis plasmid pTH1030 (Lereclus and Arantes, 1992). The following replication-related features are shared by the minimal replication regions of pLS20 and pTH1030: (i), size of the minimal replication regions (~ 1 kb); (ii), no Rep protein encoded; (iii), presence of large imperfect repeated structures. Interestingly, plasmid-driven transcriptional activity has been shown to be necessary for pTH1030 replication (Lereclus and Arantes, 1992). Although no sequence homology was observed in the replication regions of pTH1030 and pLS20, the structural similarities between these plasmids lend support to the idea that the palindrome, possibly in conjunction with transcriptional activity, are key elements in pLS20 replication. At this moment we can not rule out the possibility that also IR 1 is involved in pLS20 replication. The central region (13 bp) of this IR consists, with one exception, of A and T residues. This resembles oriS of the HSV-1. In that case the arms of the palindrome are separated by 18 A or T's. Most likely, this A and T region is the first region to become melted in oriS. Since there are several plasmids, phages and viruses known that contain more than one origin, it is not impossible that both IR 1 and IR 4 of pLS20 constitute origins of initiation of plasmid replication. Current research is aimed at distinguishing between the scenarios described above for the initiation of pLS20 replication.

MATERIALS AND METHODS

Bacterial strains, plasmids and media . Bacterial strains and plasmids used are listed in Table 4. TY medium, used for culturing Escherichia coli and B.subtilis, contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). TY plates contained in addition 2% agar. Tetracycline and kanamycin were added to final concentrations of 10 and 50 µg/ml, respectively. When regenerating protoplasts were selected for resistance to kanamycin, the concentration of this antibiotic was increased to 150 µg/ml.

DNA techniques. 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 alkaline lysis method (Sambrook et al., 1989). pLS20 DNA was isolated from logarithmically growing B.subtilis UM4 cells using the method of Anderson and McKay (1983). DNA fragments were isolated from gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, USA). Total DNA

Table 4. Bacterial strains and plasmids

Strains	Relevant properties	Source or reference
B.subtilis		
8G5	trpC2, his, met, tyr-1, ade, nic, ura, rib	Bron and Venema, 1972
PSL1	leuA8, arg15, thrA, recA4, r^-_M m^-_M	Ostroff and Pène, 1983
UM4	pLS20	Koehler and Thorne, 1987
E.coli		
JM101	thi, Δ(lac-proAB) [F' proAB $lacI^q$ lacZΔM15]	Messing, 1979

Plasmids	Properties	Reference
pTC1	pSC101-based E.coli vector with Tc^R gene of pMV158	This study
pEBSK	pBSK with Em^R gene of pE194	Kiewiet et al., 1993b
pKM1	pUC7 containing Km^R gene from pJH1	Kiel et al., 1987
pUC18	high-copy number E.coli vector containing MCS, Ap^R	Yanish-Perron et al., 1985
pMTL25	high-copy number E.coli vector with large symmetrical MCS, Ap^R	Chambers et al., 1988
pKM2	pMTL25 derivative containing Km^R gene from pKM1, Ap^R, Km^R	This study
pLS20	≈ 55 kb endogenous B.subtilis plasmid	Koehler and Thorne, 1987
pXO503	pLS20::Tn917 derivative, Em^R	Koehler and Thorne, 1987
pTLS12	pTC1 containing a 12 kb Sau3A fragment of pLS20	This study
pTLS6	pTC1 containing a 6 kb PstI fragment of pLS20	This study
pTLS6 Δ4	Deletion derivative of pTLS6; containing a 2.9 kb PstI/Eco47^{III} fragment of pLS20	This study
pTLS6 Δ2	Deletion derivative of pTLS6; containing a 4.5 kb PstI/SphI fragment of pLS20	This study
pTLS6 Δ5	Deletion derivative of pTLS6; containing a 1.5 kb PstI/SmaI fragment of pLS20	This study
pTLS6 Δ4.5	Deletion derivative of pTLS6; containing a 2 kb Eco47^{III}/StuI fragment of pLS20	This study
pEBS4	pEBSK containing a 4 kb BamHI/SmaI fragment of pLS20	This study
pWKM 1 - 9	pKM2 with various fragments of pLS20	This study

Abbreviations used: Tc^R, Em^R, Km^R and Ap^R indicate the presence of, respectively, the following antibiotic

lysates were prepared as described before (Meijer et al., 1995b). Southern transfers to Gene Screen plus membranes were carried out as described (Meijer et al., 1995c). Probe labelling, DNA hybridization conditions and washing steps were performed using the enhanced chemiluminescence DNA labelling and detection system (Amersham International, Amersham, UK). DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden). Double-stranded plasmid DNA was used as template. [^{35}S]dATP (8 μCi/μl; > 1000 Ci/mmole) was obtained from Amersham International. DNA sequences and deduced amino acid sequences were analyzed using version 6.7 of the PCGene Analysis Program (Intelligenetics Inc., Mountain View, CA). The FASTA algorithm of Lipman and Pearson (1985) was used for protein comparisons in the Swiss protein and genomic DNA databank sequences (release January 1995; MIPS, Martinsried, FRG) and the EMBL Nucleotide Sequence Database. The RDF2 program was used to evaluate sequence similarities (Pearson, 1990). To calculate z values, the KTUP value was set at 2, and 500 random shuffles of the test sequences were performed. Alignments with z values greater than 6 were considered significant; alignments with z values below 3 were considered insignificant.

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).

Plasmid maintenance assay. Plasmid maintenance was determined as described before (Meijer et al., 1995b). Briefly, overnight cultures grown in selective media were diluted 100,000 fold in non-selective media after which the percentage of plasmid-containing cells (that is: the percentage of antibiotic-resistant cells) was determined as a function of time. Since pLS20 does not contain a selectable marker, the maintenance of this plasmid was studied using pXO503; a pLS20 derivative containing a copy of Tn917 which provides the plasmid with an erythromycin resistance marker (Koehler and Thorne, 1987). In a second assay of pLS20 maintenance, ten ml of TY medium was inoculated from a single colony of B.subtilis UM4 (pLS20) cells and the culture was incubated at 37°C. Logarithmic growth was maintained by diluting the culture after approximately every 10 generations into fresh prewarmed TY medium. After 100 generations of growth, appropriately diluted samples were plated on TY agar. After overnight incubation at 37°C, the plasmid content of fifty randomly chosen single colonies was examined. For this purpose, 2 ml cultures from separate colonies were grown to late logarithmic phase; the cells were harvested and used for the extraction of total DNA. The presence or absence of pLS20 in the DNA extracts was studied by (i), electrophoresis in 0.6% agarose gels (covalently closed circular pLS20 DNA migrates more slowly than the fragmented chromosomal); and (ii), PCR reactions with the pLS20-specific primers 3 and 6 (Table 2).

PCR techniques. PCR was carried out essentially as described by Innis and Gelfand (1990). The proofreading-proficient Vent DNA polymerase (New England Biolabs, Beverly, USA) was used throughout. Template DNA was denatured for 1 min at 94 °C. Next, primers (Table 2) 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).

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