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

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ABSTRACT

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 between two divergently transcribed genes, denoted *orfA* and *orfB*, neither of which is required for replication. *orfA* shows homology to the *B.subtilis* chromosomal genes *rapA* (*spo0L*, *gsiA*) and *rapB* (*spo0P*). The 1.1 kb fragment, which is characterized by the presence of several regions of dyad symmetry, contains no open reading frames 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 1–3). Based on their mode of replication, plasmids can be divided into two groups: the first group replicates according to the rolling circle mechanism (RCM), the second according to the theta mechanism. Most small plasmids (smaller than ~12 kb) from Gram-positive bacteria use RCM; 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 RCM 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 (4–10).

Apart from a 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 in them. The currently known prokaryotic theta plasmids can be classified into four groups (11). 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 pAMβ1 (3,11–13) and the highly related streptococcal plasmids pIP501 (14–17) and pSM19035 (18,19), 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 (20), isolated from various lactococcal strains (21). Although it is known that some industrial strains of *B.subtilis* harbor large plasmids (22–24), which probably use the theta mechanism of replication, none of these has been analyzed in detail.

To extend our knowledge of the replication mechanism of theta replicating plasmids from *B.subtilis* we analyzed the replication region of plasmid pLS20. Based on its size, ~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' organism, vectors based on endogenous plasmids from *B.subtilis* are good candidates for the development of food-grade cloning systems in this organism.

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Properties	Source or reference
Bacillus subtilis		
8G5	*trpC2, his, met, tyr-*1, *ade, nic, ura, rib*	(61)
PSL1	*leu*A8, *arg*15, *thr*A, *rec*A4, r^-_M m^-_M	(62)
UM4	pLS20	(25)
Escherichia coli		
JM101	*thi*, Δ(*lac-pro*AB) [F *pro*AB *lac*I^q *lac*ZΔM15]	(63)
Plasmids		
pTC1	pSC101-based *E.coli* vector with Tc^R gene of pMV158	This study
pEBSK	pBSK with Em^R gene of pE194	(51)
pKM1	pUC7 containing Km^R gene from pJH1	(37)
pUC18	High copy number *E.coli* vector containing MCS, Ap^R	(64)
pMTL25	High copy number *E.coli* vector with large symmetrical MCS, Ap^R	(36)
pKM2	pMTL25 derivative containing Km^R gene from pKM1, Ap^R, Km^R	This study
pLS20	≈55 kb endogenous *B.subtilis* plasmid	(25)
pXO503	pLS20::Tn*917* derivative, Em^R	(25)
pTLS12	pTC1 containing a 12 kb *Sau*3A fragment of pLS20	This study
pTLS6	pTC1 containing a 6 kb *Pst*I fragment of pLS20	This study
pTLS6Δ4	Deletion derivative of pTLS6; containing a 2.9 kb *Pst*I–*Eco*47^{III} fragment of pLS20	
pTLS6Δ2	Deletion derivative of pTLS6; containing a 4.5 kb *Pst*I–*Sph*I fragment of pLS20	
pTLS6Δ5	Deletion derivative of pTLS6; containing a 1.5 kb *Pst*I–*Sma*I fragment of pLS20	
pTLS6Δ4.5	Deletion derivative of pTLS6; containing a 2 kb *Eco*47^{III}–*Stu*I fragment of pLS20	
pEBS4	pEBSK containing a 4 kb *Bam*HI–*Sma*I 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 the following antibiotic resistance genes, tetracyclin, erythromycin, kanamycin and ampicillin; MCS, multiple cloning site.

pLS20 was originally identified together with an RCM plasmid, pLS19, in *B.subtilis* strain IFO3335 (var. *natto*) (23). Koehler and Thorne (25) 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 cannot be classified in any one of the known groups of plasmids (11) and should be considered as a novel type of replicon.

MATERIAL AND METHODS

Bacterial strains, plasmids and media

Bacterial strains and plasmids used are listed in Table 1. 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. Tetracyclin 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.* (26). Restriction enzymes were obtained commercially and used as indicated by the suppliers. Plasmid DNA was isolated by the alkaline lysis method (26). pLS20 DNA was isolated from logarithmically growing *B.subtilis* UM4 cells using the method of Anderson and McKay (27). DNA fragments were isolated from gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, USA). Total DNA lysates were prepared as described before (9). Southern transfers to Gene Screen plus membranes were carried out as described (28). Probe labeling, DNA hybridization conditions and washing steps were performed using the enhanced chemiluminescence DNA labeling and detection system (Amersham International, Amersham, UK). DNA sequences were determined by the dideoxy chain termination method (29) 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/mmol) 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., Geel, Belgium). The FASTA algorithm of Lipman and Pearson (30) was used for protein comparisons in the Swiss protein and genomic DNA databank sequences (release January 1995; MIPS,

Martinsried, Germany) and the EMBL Nucleotide Sequence Database. The RDF2 program was used to evaluate sequence similarities (31). 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 >6 were considered significant; alignments with z values <3 were considered insignificant.

Transformation of *B.subtilis* and *E.coli*

Competent cells and protoplasts of *B.subtilis* were prepared and transformed as described (7,32). $CaCl_2$-treated *E.coli* cells were transformed as described by Sambrook *et al.* (26).

Plasmid maintenance assay

Plasmid maintenance was determined as described before (9). Briefly, overnight cultures grown in selective media were diluted 100 000-fold in non-selective media, after which the percentage of plasmid-containing cells (i.e. 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 Tn*917*, which provides the plasmid with an erythromycin resistance marker (25). In a second assay of pLS20 maintenance 10 ml TY medium was inoculated from a single colony of *B.subtilis* UM4 (pLS20) cells and the culture incubated at 37°C. Logarithmic growth was maintained by diluting the culture after every ~10 generations into fresh pre-warmed 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 50 randomly chosen single colonies was examined. For this purpose 2 ml cultures from separate colonies were grown to late logarithmic phase, the cells harvested and used for 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 fragmented chromosomal DNA); (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 (33). The proof-reading-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 s, 94°C), primer annealing (1 min, 50°C) and DNA synthesis (3 min, 73°C).

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 (34), which contains the multiple cloning site of pUC8. The tetracyclin 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 *Bam*HI site was first introduced using PCR techniques into pMV158 upstream of the promoter of the Tc^R gene (position 1555 according to 35). Next, the 1.6 kb *Bam*HI–*Eco*RI fragment of pMV158 containing the Tc^R gene was cloned into the corresponding sites of pHSG575, resulting in pTC1.

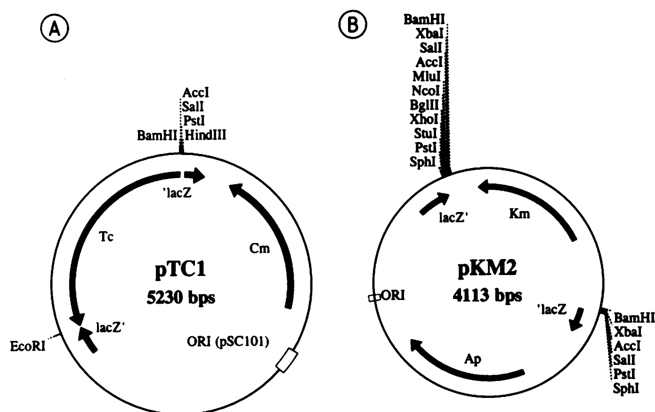

Figure 1. Schematic representation of the *E.coli* vectors used. (**A**) The low copy number vector pTC1 based on the replication functions of pSC101 and (**B**) the high copy number plasmid pKM2 based on pMTL25. Relevant restriction sites are indicated; lacZ′ and ′lacZ indicate the 5′ and 3′ regions of the *lacZα* gene; ORI, origin for replication in *E.coli*; Tc, tetracyclin resistance marker; Km, kanamycin resistance marker; Cm, chloramphenicol resistance marker; Ap, ampicillin resistance marker.

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 (36), into which the kanamycin resistance gene (Km^R) from the *Streptococcus faecalis* plasmid pJH1 was cloned. For this purpose the 1.3 kb *Hin*dII fragment of pKM1 (37), containing the Km^R gene, was cloned into the *Sma*I sites of pMTL25, resulting in pKM2. Additional advantages of this construction were that the multiple cloning site of pMTL25 still contained unique sites for 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 *Bam*HI–*Sma*I fragment, resulting in pTLS6Δ5; the 2.2 kb *Bam*HI–*Sph*I fragment, resulting in pTLS6Δ2; the 3.9 kb *Sal*I–*Eco*47^{III} fragment, resulting in pTLS6Δ4. Plasmid pEBS4 was constructed by cloning the 4 kb *Bam*HI–*Sma*I fragment of pTLS6 in a pBluescript vector containing the erythromycin resistance marker of the *Staphylococcus aureus* plasmid pE194 (pEBSK; 20).

RESULTS

Cloning of the minimal replicon of pLS20

In order to clone the replication region of pLS20 the plasmid was partially digested with *Sau*3A. After agarose gel electrophoresis fragments ranging from ~1 to 15 kb were isolated and ligated to *Bam*HI-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 ~12 kb, indicating that this fragment contained the infomation required for replication in *B.subtilis*. One of the recombinant plasmids, designated pTLS12, was used for further analysis. A restriction map of the 12 kb insert is shown in Figure 2. The replication region was further delineated by subcloning *Pst*I fragments of the 12 kb insert into the unique *Pst*I site of pTC1. Only plasmids

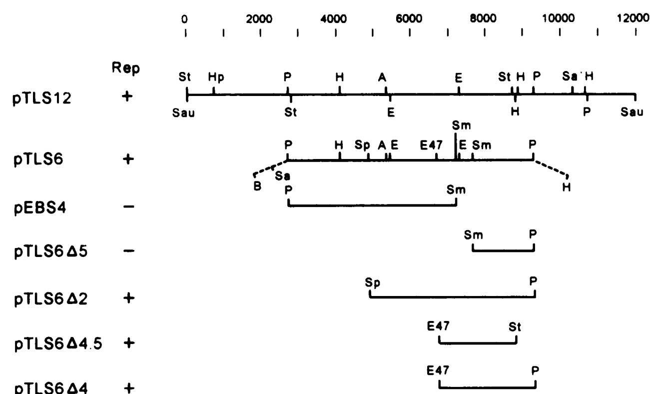

Figure 2. Schematic representation of the 12 kb region of pLS20 containing the replication functions and several deletion derivatives. + and – indicate the ability or inability to replicate in *B.subtilis*. The restriction sites at the borders of pTLS6 (shown as interrupted lines, B and Sa on the left and H on the right) represent sites originating from the multiple cloning site of the pTC1 vector. Only relevant restriction sites are indicated. Sau, *Sau*3A; St, *Stu*I; Hp, *Hpa*I; P, *Pst*I; H, *Hin*dIII; A, *Ava*I; E, *Eco*RI; Sa, *Sal*I; B, *Bam*HI; E47, *Eco*47^{III}; Sm, *Sma*I; Sp, *Sph*I.

containing the largest *Pst*I fragment of ~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 *Eco*RI 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 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 *Pst*I–*Eco*47^{III} fragment of pLS20. In addition, from the observation that pTLS6Δ5 cannot replicate in *B.subtilis* it is clear that an essential region for replication is located on the 0.95 kb *Eco*47^{III}–*Sma*I fragment, which is present in pTLS6Δ4, but absent from pTLS6Δ5. pTLS6Δ4.5, also shown in Figure 2, was constructed after the sequence of the 2.9 kb *Eco*47^{III}–*Pst*I fragment had been determined and this plasmid will be discussed in a subsequent section.

Sequence of the 2.9 kb *Pst*I–*Eco*47^{III} fragment containing the pLS20 replication functions

The 2.9 kb *Pst*I–*Eco*47^{III} 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 Figure 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 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 >100 amino acids, each containing a putative start codon preceded by a potential ribosomal binding site (RBS). These ORFs, designated *orf*A and *orf*B, are divergently transcribed relative to each other. Several properties of these *orfs* are summarized in Table 2. The frame of *orf*B is still open at the *Eco*47^{III} site, which defines the end of the cloned fragment. Therefore, in the sequence shown *orf*B is truncated after codon 110. The deduced *orf*A product has 45% similarity with two chromosomally encoded *B.subtilis* protein-aspartate phosphatases: *rap*A (originally identified as a glucose starvation-induced gene, *gsi*A; 38; also named *spo*OL) and *rap*B (formerly named *spo*OP; 39). *Rap* stands for response regulator aspartate phosphatase (39). The chromosomal *rap*A gene (called *gsi*AA in 38) is followed by a translationally coupled small gene, *gsi*AB, specifying 44 amino acids. *orf*A of pLS20 is also characterized by the presence of a translationally coupled ORF, which we will designate *orf*AB. Due to the cloning procedure, *orf*AB was truncated after codon 21 in pTLS6Δ4. The DNA sequence of the complete *orf*AB was determined, however, by sequencing 250 bp downstream of the *Pst*I site using pTLS12 as template (bp 2881–3136 in Fig. 3). The additional sequence data showed that, like *gsi*AB, *orf*AB also specified a putative protein of 44 amino acids (Table 2). Directly downstream of *orf*AB is an inverted repeat structure, which is likely to function as a rho-independent transcriptional terminator (Fig. 3). In all these aspects the structural organization of the pLS20 *orf*A and *orf*AB genes resembles that of the chromosomal *rap*A genes. In contrast to *orf*A, no significant homology was observed between the truncated *orf*B and available sequences in databanks.

Table 2. Properties the *orf*A, *orf*B and *orf*AB genes

Gene	Position[a]	Size[b]	Mass (kDa)[c]	Putative start codon and RBS[d]	Spacing (bp)[e]
*orf*A	1720–2823	368	43.4	ttgaatagctggagggaagtgtATG	9
*orf*AB	2823–2954	44	4.6	aaagaataacggaggcgttaggATG	9
*orf*B	332–1 (C)[f]	> 110		aatcaaaggagagaataaaaatATG	12

[a] The numbers correspond to positions in Figure 3.
[b] Size in codons.
[c] Molecular masses are calculated from the deduced amino acid sequences.
[d] 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, UCUUUC-CUCCACUAG (65), are underlined.
[e] 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.
[f] C indicates that the ORF is located on the opposite strand.

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ColE1 cer      GGTGCGTACAATTAAGGGATTATGGTAAAT
E.coli dif     GGTGCGCATAA TGTATA TTATGTTAAAT
pSC101 psi     GGTGCGCGCAA GATCCA TTATGTTAAAC
pLS20          GAGTTTTTTAA AAAAAA TTATGTTAAAG
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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.

The deduced products of *orf*A, *orf*AB and *orf*B do not share homology with known replication proteins. The intergenic region between *orf*A and *orf*B 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.

The following characteristic features were identified in the 1.4 kb intergenic region between *orf*A and *orf*B by computer-assisted analysis.

(i) 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 Figure 3 and their positions are indicated in Figure 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.

(ii) 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%): (a) position 1463–1487 (25 bp with 92% AT); (b) position 674–709 (35 bp with 86% AT); (c) position 442–457 (17 bp with 94% AT); (d) position 387–407 (19 bp with 85% AT). Third, several (imperfect) direct repeats were identified (Fig. 3). The direct repeats are: (a) 5′-AAAATGAAATCA-3′ (starting at positions 397 and 453); (b) 5′-AAATCAGTGAA-3′ (starting at positions 414 and 459); (c) 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 673–708) contains the nearly identical sequence 5′-ACAAATAAAAAG-3′ three times.

(iii) Several sequences were identified showing homology to DNA sequences known to be involved in replication. (a) DnaA boxes were identified starting at the following positions (coordinates according to Fig. 3): 1038 (consensus DnaA box, 5′-TTATCCACA-3′, lower strand); 998 (two mismatches compared with consensus, 5′-TTcaCCACA-3′, lower strand); 1029 (three mismatches compared with consensus, 5′-TTcTCCggA-3′, lower strand); 960 (three mismatches compared with consensus, 5′-TataCCACA-3′). All potential DnaA boxes are located within the region of low AT content. (b) Upstream of *orf*A (position 1626–1644) 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.subtilis* chromosomal DNA terminator (40). 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). (c) A short DNA sequence of 10 bp within the coding sequence of *orf*A was identified (position 2037–2046 in Fig. 3, 5′-TTATGTTAAA-3′) which is identical to part of the *E.coli* chromosomal XerCD recombination site (*dif*) (41) and the equivalent *psi* (42) and *cer* sites (43) of *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 *orf*A) was still able to replicate in *B.subtilis*, despite the fact that *orf*B and *orf*A 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 *orf*A and *orf*B 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 3, are indicated in Figure 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 *orf*A and *orf*B. Primers 3 and 6 were located ~150 bp upstream of these RBS sequences. Nine regions were amplified 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 *Bam*HI site (not present in the *orf*A–*orf*B intergenic region). The amplified PCR products were digested with *Bam*HI and ligated to *Bgl*II-linearized pKM2 DNA. The ligation mixtures were used to transform *B.subtilis* PSL1 protoplasts. Kanamycin-resistant transformants were obtained with six 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

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 a 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 start codon, are present between the divergently transcribed *orf*A and *orf*B: (i) lower strand, position 727–536, MLQHRTLFIC-HSLFVFNFAN-SSQTKYVSSN-LLTLLSANNR-VFCCLFRITR-TRIQCQNFHI-DLT; (ii) upper strand, position 998–1246, MARTAINGPE-NVDKPLGCLA-HYGAGNYGQA-SMQRHTIECD-RVREHETNPI-EVAYRTSIVC-KKVMAGESLA-IARQSWKPQT-SL; (iii) upper strand, position 1243–1395, MTLVSNPDRL-PMSVRFYFLR-DRHRQSKFAL-FPRTVSYSAS-FKFSKSREKH; (iv) upper strand, position 759–923, MFKLCLHFTE-NKCIRQHFSA-IFGRQKTTVW-RVSNRGFLSF-FVCHPAAVKG-CKIN.

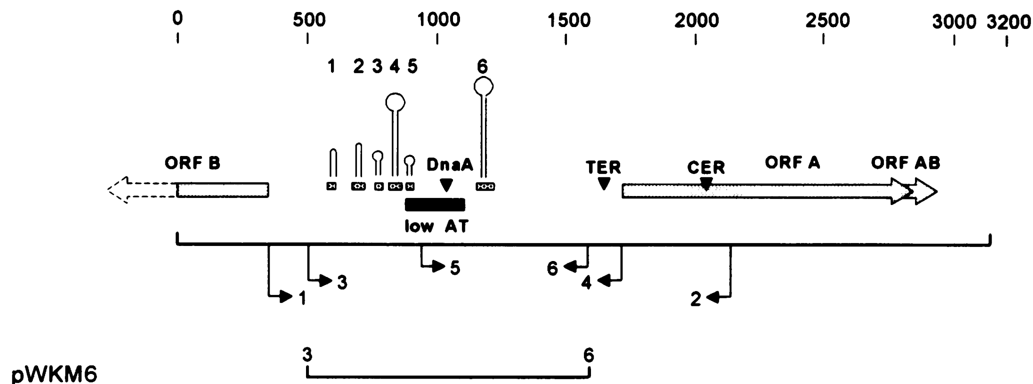

Figure 5. Schematic representation of the pLS20 replication region. The positions of the primers used for amplification of the various regions of pLS20 are indicated. pWKM6 is a pKM2 derivative containing the indicated pLS20 region. The following characteristics are shown: *orf*A, *orf*AB and *orf*B (arrows); consensus DnaA box (DnaA); DNA replication terminator (Ter); cer-like site (cer) (the latter three characteristics are indicated with triangles). The six regions of dyad symmetry are numbered 1–6. 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*.

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 *Pst*I (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 *orf*A and *orf*B have not been mapped, it is likely that these are not present in pWKM6, since a region of ~150 bp upstream of the 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 number were observed between the various constructs. As judged from ethidium bromide stained agarose gels the constructs had a copy number of 2–4 per chromosome equivalent (data not shown).

The observation that the three constructs containing the pLS20 region 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 *Sma*I fragment (positions 491–936 in Fig. 3). This is in agreement with the observation that pTLS6Δ5, also lacking this *Sma*I fragment, was not able to replicate in *B.subtilis*. Characteristics identified in this 0.44 kb *Sma*I region are five of the six identified regions with dyad symmetry and the AT-rich region containing the nearly identical repeat 5′-CTTTTTATTT-GT-3′ three times.

Table 3. Primers used

Name	Position[a]	Sequence (5′→3′)[b]
LS20-1	345 →	ggggaTCCTTTGATTTCACTTTCCTC
LS20-2	2139 ←	ggggaTCCAATCTTATAGTGAAACTCCGC
LS20-3	491 →	ggggatcCAGG**CCCGGG**GCTTTACGTC
LS20-4	1722 ←	ggggatcCAACATACACTTCCCTCCAGCT
LS20-5	936 →	ggggatCCCCGTCCACCGCAAA**CCCGGG**G
LS20-6	1592 ←	ggggatCCCAAGCATGTACTGATATC

[a]Positions are according to Figure 3, directions of primers are indicated with arrows.
[b]Nucleotides identical to pLS20 sequences are in upper case letters. Lower case letters represent 5′ extensions specifying *Bam*HI sites. *Sma*I sites are shown in bold.

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 (Pol I) enzyme; (v) effects of prevention of translation on plasmid copy number.

(i) Maintenance of pLS20 miniderivatives. Although pLS20 has a low plasmid copy number (1–3 per chromosome; 25), it is maintained very stably. Plasmids with low copy numbers generally encode functions to ensure their stable maintenance (44,45). 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 ~25% of cells harbored 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 the 12 kb region present in pTLS12.

(ii) Production of ssDNA replication intermediates. A major distinction between RCM and theta replication is the production of ssDNA replication intermediates in the former system. To study the possibility that pLS20 uses RCM replication we analyzed whether pLS20 miniderivatives produced ssDNA. For this purpose total DNA extracts were prepared from *B.subtilis* PSL1 cells harboring either the pLS20 derivatives pTLS12 or pTLS6 or the RCM plasmid pLS1, which is known to accumulate ssDNA in *B.subtilis* (10). 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 RCM replication.

(iii) Thermoresistance of pLS20 replication. Although the theta replicating plasmid pAMβ1 originally isolated from *Streptococcus faecalis* is able to replicate in *B.subtilis*, its replication is thermosensitive in this bacterium (46). Also, replication of the *B.thuringiensis* theta plasmid pTH1030 was reported to be thermosensitive in *B.subtilis* (47). Vectors based on these plasmids cannot replicate at 47°C and, therefore, cannot 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 on the host-encoded Pol I enzyme. To investigate whether replication of pLS20 depends on functional Pol I plasmids pWKM1–6 were tested for their ability to transform the *pol*A5 *B.subtilis* strain 1A226 and the isogenic Pol I-proficient strain 1A224. The *pol*A5 strain 1A226 lacks Pol I, which was shown by genetic, biochemical and partial sequence analyses (11,48,49). Whereas RCM replicating plasmids, like pUB110, can replicate in the *pol*A5 strain, the theta replicating derivative pIL252 of plasmid pAMβ1 is unable to do so (11). Therefore, these plasmids were used as positive and negative controls for pLS20 derivatives. The results, presented in Table 4, show that all pLS20 derivatives tested transformed both the *pol*A5 strain and the isogenic Pol I-proficient strain efficiently to kanamycin resistance. As expected, the control plasmids pUB110 and pIL252 transformed the Pol I-proficient strain efficiently to antibiotic resistance, whereas only pUB110 also transformed the *pol*A5 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 chloramphenicol. After the addition of 100 μg/ml chloramphenicol to cultures of logarithmically growing cells harboring either pWKM2 or pWKM3 samples of the cultures were taken as a function of time and the plasmid copy number was determined. No increase in copy number was observed even at 4 h after addition of chloramphenicol (results not shown).

Table 4. Pol I independence 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

DISCUSSION

Two modes of DNA replication are known for bacterial plasmids: the RCM and the theta mechanism. Plasmids replicating according to RCM are easily recognized by: (i) their limited size (<12 kb); (ii) the generation of ssDNA replication intermediates; (iii) the presence of several functional modules. Examples of these modules are a *rep* gene encoding the essential replication initiator protein and modules comprising the initiation sites for leading and lagging strand synthesis (for a review see 1). None of these typical features of RCM plasmids apply to pLS20: (i) pLS20 is large (~55 kb); (ii) no ssDNA intermediates were detected; (iii) no characteristic modules of RCM plasmids were identified. These data indicate that pLS20 uses the theta mechanism rather than RCM 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; (iii) a replication terminator (Meijer *et al.*, in preparation). Although these properties are not absolute proof, they 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 >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 of how replication of pLS20 is initiated. The currently known theta plasmids can be classified into four groups, designated A–D (11). This classification is based on three characteristics: the presence or absence of a Rep protein; the presence of an *ori*A-like structure; the dependence of replication on host-encoded Pol I. Class A includes plasmids which encode a replication protein (Rep) and these plasmids have a characteristic replication origin, designated *ori*A (50). Typically, *ori*A contains repeated sequences (iterons) recognized by the cognate Rep protein, one or more DnaA boxes and an AT-rich region which generally contains repeats. Pol I is not required for this type of 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 (21,51), are likely to belong to this class. Class B replicons do not encode a Rep protein and lack the typical *ori*A region (50). 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 Pol I and later taken over by the replication enzyme complex (50). ColE1 is the archetype of this family of plasmids. Class C contains a group of at least 17 related plasmids, collectively called ColE2-related plasmids (52). These plasmids encode a Rep protein essential for replication (53–55) and require host-encoded Pol I (56,57). 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 (55), and synthesize a unique primer RNA (ppApGpA) which is used for initiation of leading strand synthesis by Pol I (58,59). Like class C plasmids, class D plasmids, represented by the *S.faecalis* plasmid pAMβ1, encode a Rep protein and require Pol I for initiation of replication. Although an *ori*A-like structure was identified upstream of the *rep* gene, this structure is not required for replication (11). 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 class C plasmids.

pLS20 cannot be classified in any 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 Pol I mutated strain and its copy number was not affected by chloramphenicol treatment. What then 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 Pol I; (ii) its replication region contains DnaA boxes and an AT-rich region with imperfect direct repeats, elements which are typical for *ori*A-like structures. The AT-rich regions within *ori*A structures of class A plasmids are believed to constitute the first regions to be melted during replication. The AT-rich region identified in pLS20 (position 674–709, Fig. 3) is located on the 0.44 kb *Sma*I fragment, which is essential for pLS20 replication. Possibly this region of pLS20 functions as the first region to be melted during replication initiation. An alternative explanation for the requirement for this *Sma*I region for replication is based on the presence of several inverted repeat 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 melt region is located in a palindromic sequence (50). In these viruses and some bacteriophages the initial melt step is affected by transcriptional activity at or near the origin (50). 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 (position 884–916, 5′-TTGtCA...[21 nt]...TAaAAT-3′, and position 806–778, 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 (60). 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) the presence of large imperfect repeated structures. Interestingly, plasmid-driven transcriptional activity has been shown to be necessary for pTH1030 replication (60). 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, is a key element in pLS20 replication. At this moment we cannot rule out the possibility that IR 1 is also involved in pLS20 replication. The central region (13 bp) of this IR consists, with one exception, of A and T residues. This resembles *ori*S of HSV-1. In that case the arms of the palindrome are separated by 18 A or T residues. Most likely this A and T region is the first region to become melted in *ori*S. Since there are several plasmids, phage 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 initiation of pLS20 replication.

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