Replication and maintenance of plasmids in Bacillus subtilis

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Chapter IX

Identification and characterization of a novel type of replication terminator with bidirectional activity on the *Bacillus subtilis* theta plasmid pLS20

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SUMMARY

We have sequenced and analyzed a 3.1 kb fragment of the 55 kb endogenous Bacillus subtilis plasmid pLS20 containing its replication functions. Just outside the region required for autonomous replication, a segment of 18 bp was identified as being almost identical to part of the major B.subtilis chromosomal replication terminator. Here, we demonstrate that this segment is part of a functional replication terminator. This newly identified element, designated TerLS20, is the first replication terminator identified on a Gram-positive theta plasmid. TerLS20 is distinct from other known replication terminators in the sense that it is functional in both orientations. The region required for bipolar functionality of TerLS20 was delineated to a sequence of 29 bp, which is characterized by an imperfect dyad symmetry.

INTRODUCTION

The process of DNA replication can be divided into three steps: initiation, elongation and termination. DNA termination of various theta replicons of prokaryotic origin has been studied intensively over the last two decades (for reviews see (Hill, 1992), (Yoshikawa and Wake, 1993) and (Baker, 1995). The concept of a replication terminus in Escherichia coli was first advanced by Masters and Broda (1971) and Bird et al. (1972), who concluded that bidirectional replication of the chromosome is initiated from a unique origin, oriC, and that the two replication forks meet on the opposite side of the chomosome. Later, Kuempel and coworkers (1977; 1978) and Louarn and coworkers (1977; 1979) demonstrated that clockwise and counterclockwise replication forks were arrested within a particular region of the chromosome, which was designated the terminus. Research in recent years in different laboratories has revealed that two components are required for replication arrest in E.coli. These are the Ter sites, which are non-palindromic DNA sequences of 22 bp (Hill et al., 1988), and the Tus protein, encoded by the chromosomal tus gene, which is a DNA-binding protein that recognizes and binds specifically to the Ter sequences (Hill et al., 1989). Binding of the Tus protein to a Ter sequence results in the polar arrest of replication fork movement. Six termination sites have been identified on the E.coli chromosome (TerA-TerF) which are distributed in a region comprizing approximately 25% of the total chromosome opposite oriC. Three of these sites arrest clockwise replication, and the other three arrest counterclockwise replication. Thus, moving replication forks are trapped in the relatively wide TerC region.

Replication arrest sites have also been identified in several plasmids of Gram-negative bacteria, such as R6K, R100, R1 and P307. Interestingly, the sequences of these plasmid-located terminator sites are highly similar to the chromosomal terminator sites (Horiuchi and Hidaka, 1988; Hill, 1992). Replication fork impediment of plasmids also occurs through the binding of the chromosomally-encoded Tus protein to the plasmid-located Ter sites (Sista et al., 1989). Except for the terminus

region of P307 (Saadi et al., 1987), those of the other three plasmids consist of two Ter sites which are present as an inverted repeat (Horiuchi and Hidaka, 1988; Miyazaki et al., 1988). At each site plasmid replication is arrested in an orientation-dependent manner (Horiuchi and Hidaka, 1988). Thus, the two polar Ter sequences form a replication fork trap and halt replication from either direction. Interestingly, the plasmid-located terminator sites are not located diametrically opposite of their origin of replication.

The chromosome of Bacillus subtilis also contains a terminus region which is located approximately opposite the origin of replication, oriC (Weiss and Wake, 1983; Iismaa et al., 1984). Many characteristics of the replication arrest systems are similar in B.subtilis and E.coli (for a review of the B.subtilis termination process see (Yoshikawa and Wake, 1993)). The B.subtilis terminus region contains also six terminators (TerI-TerVI) which are arranged in a similar way to those in E.coli to ensure that the approaching forks meet within a restricted region of the chromosome (Franks et al., 1995). In B.subtilis also, replication fork arrest occurs via the binding of a specific protein, RTP (Replication Terminator Protein) to the specific terminator sites.

Another feature of both organisms is the association of one of the inner terminators (TerI [formerly denoted IRI] in B.subtilis and TerB in E.coli) with the gene for the cognate terminator protein (RTP in B.subtilis, Tus in E.coli) in such a way that expression of the gene is negatively autoregulated by binding of the terminator protein (Ahn et al., 1993; Roecklein and Kuempel, 1992). However, in addition to these similarities, striking differences also exist between the B.subtilis and E.coli replication arrest systems. First, the DNA sequences of their terminator sites are different. Second, the primary structure of RTP of B.subtilis shares only limited homology with the Tus protein of E.coli (Bussiere et al., 1995). Third, the binding characteristics of the two terminator proteins to their cognate DNA sequences are different. In B.subtilis, two dimers of RTP interact with each terminator sequence and the cooperative interaction between the two dimers seems to be needed for polar fork arrest (Lewis et al., 1990; Smith and Wake, 1992). In E.coli, binding of a single Tus monomer results in the polar arrest of a replication fork (Hill et al., 1988; Hill et al., 1989; Sista et al., 1989).

Hitherto, no replication terminator sites have been identified on theta-replicating plasmids from B.subtilis. Moreover, no detailed studies concerning theta-replicating plasmids from this bacterium have been carried out sofar. We are interested in endogenous B.subtilis theta plasmids for the following reasons. First, we want to extend knowledge of the replication mechanism of theta-replicating plasmids, especially those from B.subtilis. Second, we reason 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. Therefore, we have studied the replication region of the 55 kb cryptic B.subtilis theta plasmid pLS20. This plasmid, first described by Tanaka et al. (1977), is present in the B.subtilis natto strain IFO335, together with a 5.8 kb cryptic plasmid, pLS19. Koehler and Thorne (1987) isolated a cured B.subtilis, UM4, which contained only pLS20. We have isolated pLS20 from this strain and cloned and sequenced a 3.1 kb region of this plasmid containing all the information required for autonomous replication in B.subtilis (to be published elsewhere). In this paper we describe the identification of a functional replication terminator which is located just outside the region required for autonomous replication present on the 3.1

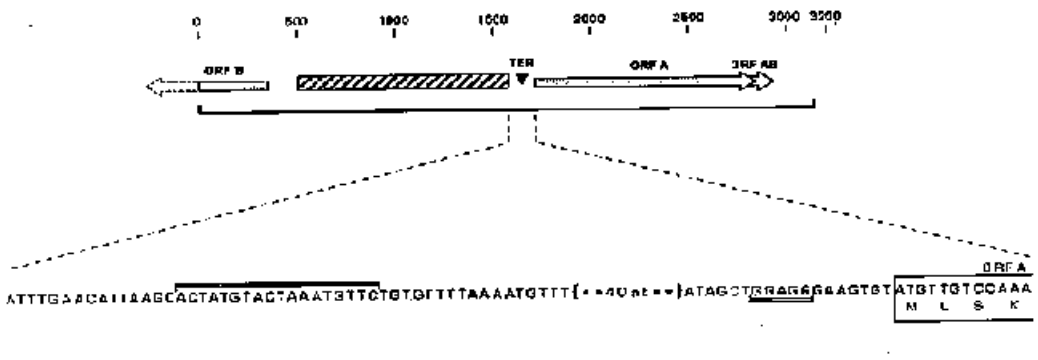

Figure 1. Organization of the 3.1 kb fragment of pLS20 containing its replication region. (Top): Genes orfA, orfAB and orfB (truncated) are indicated with arrows; the delineated region required for replication is indicated with a hatched bar and the position of TerLS20 (TER) is indicated by a filled triangle. (Bottom): Sequence of the pLS20 region containing the fragment which is homologous to part of the B.subtilis chromosomal terminators. The homologous region is double overlined. The beginning of

kb sequenced fragment of pLS20. Evidence is presented that this replication terminator is distinct from all known prokaryotic replication arrest sites in the sense that it is able to block the movement of replication forks approaching from both directions.

RESULTS

Identification of a pLS20 sequence showing homology to the B.subtilis chromosomal terminator .

We have cloned and sequenced a 3.1 kb region of the B.subtilis plasmid pLS20 containing the replication functions. A schematic representation of the structural organisation of this region is shown in Fig. 1. We were interested to know whether this region contained a functional replication terminator. Since the DNA sequences of terminator sites on the Gram-negative plasmids R6K, R100, R1 and P307 are very similar to those on the chromosome of E.coli (Horiuchi and Hidaka, 1988; Hill, 1992)), we felt that a possible replication terminator site on B.subtilis plasmids might, likewise, be homologous to the chromosomal terminator sites of B.subtilis. We therefore analyzed the sequenced 3.1 kb fragment of pLS20 for DNA sequences homologous to those of the B.subtilis chromosomal terminators TerI and TerII (the only chromosomal terminators known when this work was performed). The structural organization of the B.subtilis 168 terminus region containing the major arrest site, TerI, is presented in Fig. 2A. The inverted repeats, designated TerI and TerII are 47 and 48 basepairs long, share 77% identity and are separated by 59 nucleotides (Carrigan et al., 1987; Lewis and Wake, 1989). The equivalent terminus region of the B.subtilis strain W23 chromosome is highly conserved compared to that of strain 168 (Lewis and Wake, 1989). A region of 18 bp was identified in pLS20 which, except for 1 bp, is identical to part of TerI and TerII of B.subtilis strain 168 (Fig. 2A). The alignment between homologous regions of the chromosomal TerI through TerVI sequences and the region of pLS20 containing the identified 18 bp is shown in

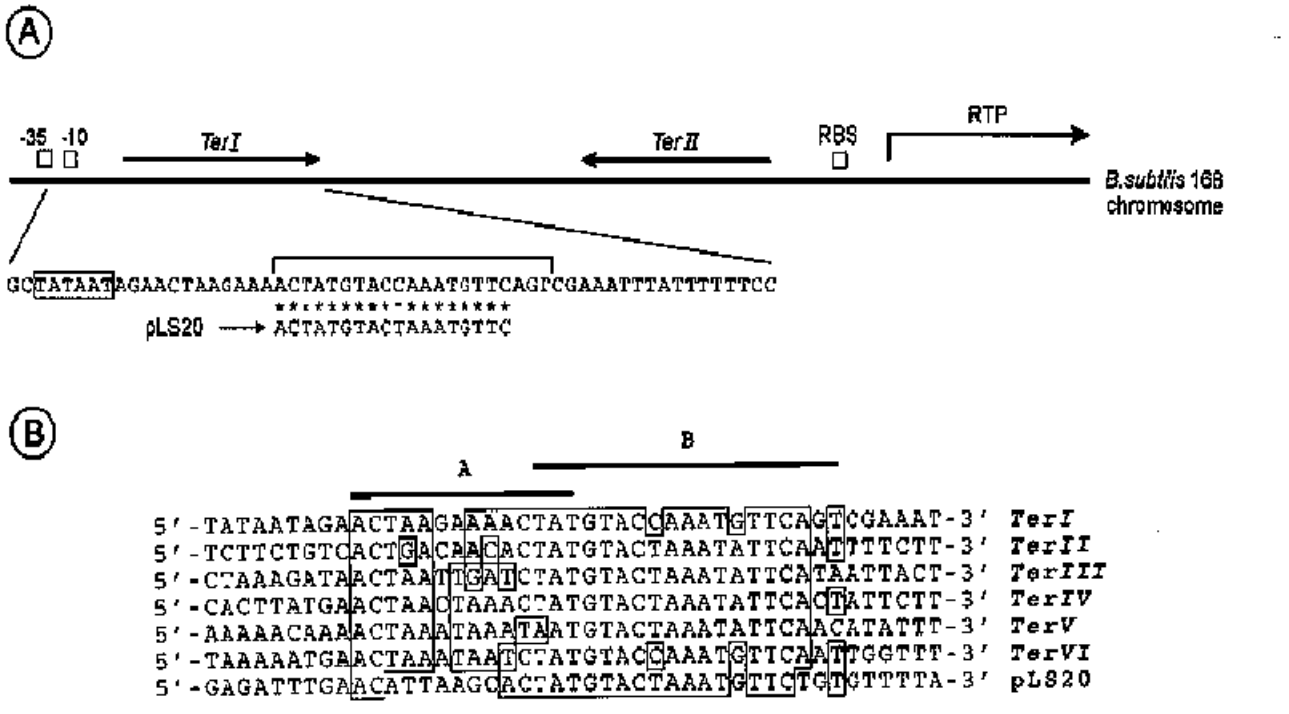

Figure 2. Homology between B.subtilis chromosomal replication terminators and a region of pLS20.
(A) Homology between the chromosomal region of B.subtilis 168 containing TerI and TerII, and the pLS20 region. The following features of the chromosomal terminus region are schematically indicated: TerI and TerII (arrows); the position of the rtp gene (RTP), its ribosomal binding site (RBS) and its promoter (-35 and -10 left of TerI). The sequence of the TerI and the homologous region of pLS20 is shown below the linear map of the terminus region. Indicated are the -10 sequence of the rtp promoter (boxed) and the 21 bp core sequence capable of binding 1 RTP dimer (overlined); Identical nucleotides are indicated with asterisks. The sequence of the chromosomal replication terminus region was taken from

Fig. 2B. The latter sequence is located 63 bp upstream of the putative ribosomal binding site of the orfA gene, which has significant homology to the B.subtilis chromosomally encoded rapA and rapB genes (Perego et al., 1994). RapA was previously denoted gsiA (Mueller et al., 1992). The comparison between the B.subtilis chromosomal terminator sequences and the homologous region of pLS20 reveals two clear differences. First, in pLS20 the inverted repeat DNA sequence as observed in the arrangement of the B.subtilis chromosomal terminators, TerI and TerII, is absent and second, the homology involves only part of the TerI and TerII sequences (Fig. 2A).

In vitro binding of RTP to the pLS20 Ter region.

The chromosomal terminators arrest a moving replication fork only when both RTP-binding sites (A and B) are bound by an RTP dimer (Smith and Wake, 1992). These partly overlapping RTP-binding sites, as determined by Langley et al. (1993), are shown above the DNA sequences in Fig. 2B. The presence of only RTP-binding site B, which has an RTP-binding affinity similar to that of TerI, is insufficient to impede a moving replication fork (Smith and Wake, 1992). Since the region of pLS20 which is homologous to the B.subtilis chromosomal terminators includes the entire RTP-binding site B but only part of binding site A, it was of

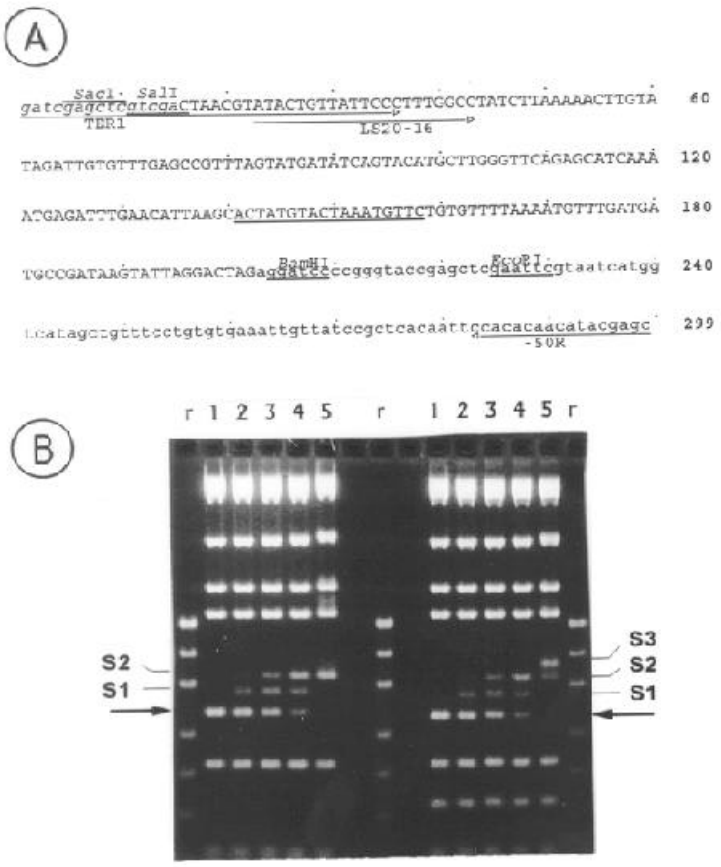

Figure 3. Band retardation assays for binding of RTP to TerI and a fragment of pLS20.
(A) DNA sequence of the fragment used in the band retardation studies (fragment 1; region between the primers LS20-16 and -50R) and the sequence of the inserts in pAB120-1 and pAB121-1 (see also Experimental Procedures). Sequences of the different primers and the relevant restriction sites used are indicated. The SacI/BamHI and SalI/EcoRI regions represent the sequences cloned in pAB120-1 and pAB121-1, respectively. The 18 bp sequence which is nearly identical to a segment of TerI is underlined. Nucleotides originating from pLS20 are shown in upper case letters; lower case letters represent nucleotides from pUC18; letters shown in lower case and italics are non-pLS20 sequences which are part of primer TER1 containing restriction sites. Sequences of primers TER1, LS16 and -50R, used for the amplification of the fragments, are indicated.
(B) Plasmid pWS66-1 was digested with PvuII + BstNI. pWS64-1 was cut with PvuII + BstNI + BamHI and then mixed with fragment 1. TerI and fragment 1 are present in the 280 bp fragment (left panel) and 277 bp fragment (right panel) indicated with the arrows. Assays were performed as described by Lewis et al. (1989) in 6.0 µl volumes with increasing ratios of RTP dimer to DNA. Band retardation was analyzed by electrophoresis in 4% Sigma wide-range agarose gels followed by ethidium bromide staining as described (Lewis and Wake, 1989). In lanes 1 through 5 the RTP dimer to DNA fragment (TerI or fragment 1) molar ratios were 0, 1, 2, 4, and 8, respectively. Lanes indicated with r show DNA standards of 501, 489, 404, 331, 242, and 190 bp. s1, s2 and s3 represent retarded DNA species.

interest to study the ability of this region to bind RTP. For this purpose, band retardation assays (Freid and Crothers, 1981) were performed using a 277 bp DNA fragment, denoted as fragment 1 (Fig. 3A), consisting of a 180 bp fragment of pLS20, encompassing the 18 bp region homologous to the B.subtilis chromosomal terminators, and a short additional region of pUC18. In this experiment, pWS66-1, which contains the TerI region of the B.subtilis chromosome was used as a positive control. The binding assays for digests of pWS64-1 plus fragment 1 (the pWS64-1 digest was

included to provide competing DNA comparable to that present in the positive control, see Materials and Methods), and for digests of pWS66-1 were performed under identical conditions. Fig. 3B shows the electrophoretic resolution of the various DNA fragments under conditions of increasing RTP dimer to DNA ratios. Lanes with identical numbers represent the same RTP dimer to DNA fragment ratios (TerI and fragment 1). As expected from previous work (Lewis et al., 1990), the positive control with the TerI fragment (Fig. 3B; left panel) gave rise to two retarded DNA species, s1 and s2, at intermediate RTP levels, reflecting the presence of the two binding sites (A and B) for RTP. Each of these sites binds a dimer of RTP (Lewis and Wake, 1989). Approximately 50% of the TerI fragment is converted to retarded species in lane 3 (ratio RTP dimer to DNA fragment ≈ 2). Saturation of both binding sites does not occur until the ratio of RTP dimer to DNA is about 8 (left panel, lane 5). In the case of fragment 1 of pLS20 (Fig. 3B; right panel), a similar retardation pattern was observed (compare lanes 2, 3 and 4 of the left and right panels). These

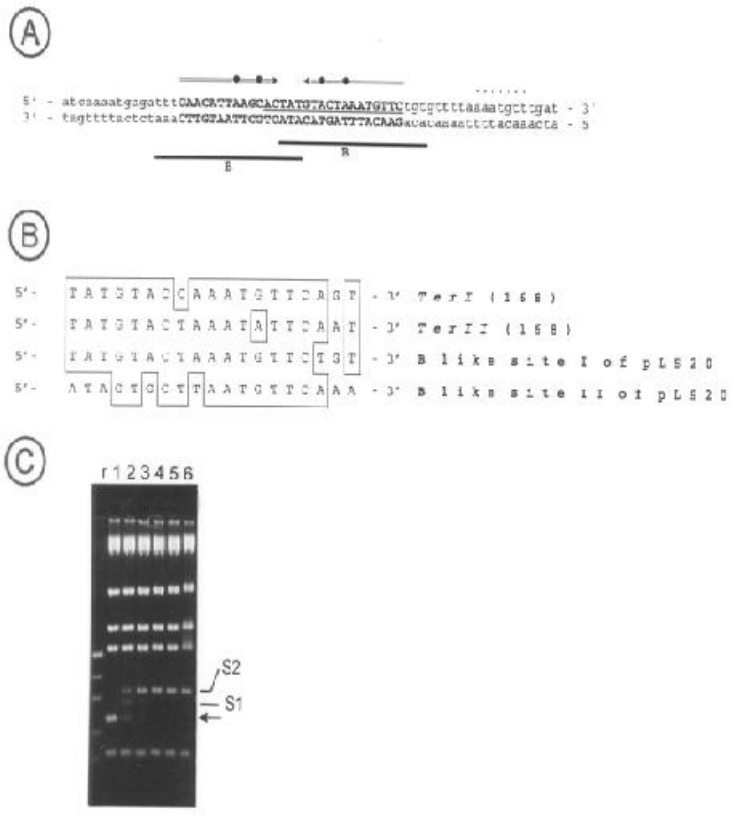

Figure 4. Inverted repeat sequence in pLS20 with putative RTP-binding sites B and its RTP-binding capacity. (A) Schematic representation of the inverted repeat. The 18 nucleotides which are nearly identical to nucleotides of TerI are underlined. The inverted repeat structure is indicated with arrows (filled circles indicate mismatches). Bars indicate the regions that are homologous to RTP-binding site B as determined for TerI of B.subtilis strain 168 (Langley et al., 1993). The three nucleotides (GTT) at which the replication fork is arrested largely when the leading strand reaches this site in TerI (Bruand et al., 1991), is present in each half repeat (indicated with brackets). The heptamer sequence (5'-AAATGTT-3'), which is an internal sequence of the RTP-binding site B, is also present just outside the inverted repeat structure (indicated with dots). (B) Alignment of the two half repeats of IR-LS20 with the B.subtilis chromosomal RTP binding sites B of strain 168 (TerI - TerVI). Identical nucleotides are boxed. (C) Band retardation assay for binding of RTP to the 29 bp imperfect inverted repeat of pLS20 shown in (A). Plasmid pWS95-1 was digested with PvuII + BstNI. The 29 bp inverted repeat is present in the 279 bp fragment indicated with the arrow. Assays were performed as described in Fig. 3B. In lanes 1 through 6 the RTP dimer to DNA fragment (imperfect inverted repeat) molar ratios were 0, 1, 2, 4, 8 and 16, respectively. s1 and s2 represent retarded DNA species.

results clearly indicate that fragment 1 is able to bind two RTP dimers at intermediate RTP levels and that the binding properties of these two RTP dimers to fragment 1 are similar as those of the chromosomal TerI region. Like TerI, saturation of two RTP-binding sites is reached when the ratio is 8. However, in contrast to the results obtained with TerI, a third retarded species, s3, was observed with fragment 1 under these conditions (Fig. 3B; right panel, lane 5). Since only the RTP-binding site B of TerI is highly conserved in pLS20, binding of only one RTP dimer was anticipated. A possible explanation for the binding of the second and, under high RTP to fragment ratios, even a third RTP dimer was obtained by re-examining the putative terminator region of pLS20. The conserved 18 nucleotides of pLS20, which are homologous to chromosomal TerI sequences, are part of an almost perfect inverted repeat of 29 nucleotides, designated IR-LS20, which consists of two half-repeats of 13 nucleotides separated by 3 nucleotides (Fig. 4A). Each of the half repeats has considerable homology with the chromosomally-located RTP-binding site B (Fig. 4B). This suggested to us that the identified IR-LS20 might contain the two RTP-binding sites on fragment 1 which were observed at intermediate RTP dimer to DNA ratios. To test this idea, the 29 bp fragment comprizing IR-LS20 was cloned in both orientations into pWS64-1, resulting in plasmids pWS98-1 and pWS95-1. Band retardation studies, performed with appropriate restriction digests of pWS98-1 and pWS95-1 showed that the fragment containing IR-LS20 was indeed able to bind two RTP dimers. The result for pWS95-1 is shown in Fig. 4C. As observed for fragment 1, intermediate levels of RTP resulted in two retarded species and saturation was observed at an RTP dimer to fragment ratio of about 8. In contrast to fragment 1, no additional third retarded species was observed with the 29 bp fragment even under conditions of ≈ 16 RTP dimers to DNA (Fig. 4C, lane 6). Possibly, the third RTP-binding site observed with fragment 1 at high RTP/DNA ratios is associated with the presence of the sequence 5'-AAATGTT-3' which is located just outside the IR-LS20 region (indicated in Fig. 4A). This 7-mer is an internal sequence of the RTP-binding site B.

In vivo arrest of replication fork movement by the pLS20 replication terminator.

The Ter sequences of the B.subtilis chromosomal terminus region cause polar termination of replication fork movement in vivo under conditions in which RTP is overproduced (Smith and Wake, 1992). In these experiments, TerI and TerII were cloned separately in both orientations in the high-copy number E.coli/B.subtilis shuttle plasmid pWS64-1, which is based on pIL253 (a 4.96 kb high-copy number derivative of pAMß1) and a derivative of pGEM3Zf(+) (Smith and Wake, 1992) (Fig. 5A). In B.subtilis, replication of pWS64-1 starts from the pAMß1 origin, the position of which is known (Bruand et al., 1991), and proceeds unidirectionally as indicated (filled triangle) in Fig. 5A. Replication continues until the migrating fork encounters a functional terminator, which causes transient fork arrest and results in the accumulation of Θ-shaped replicative intermediates. Using Southern hybridization, replication intermediates can be detected. This technique was exploited to establish whether the region of pLS20, to which RTP specifically binds, is able to arrest a migrating replication fork in vivo. For this

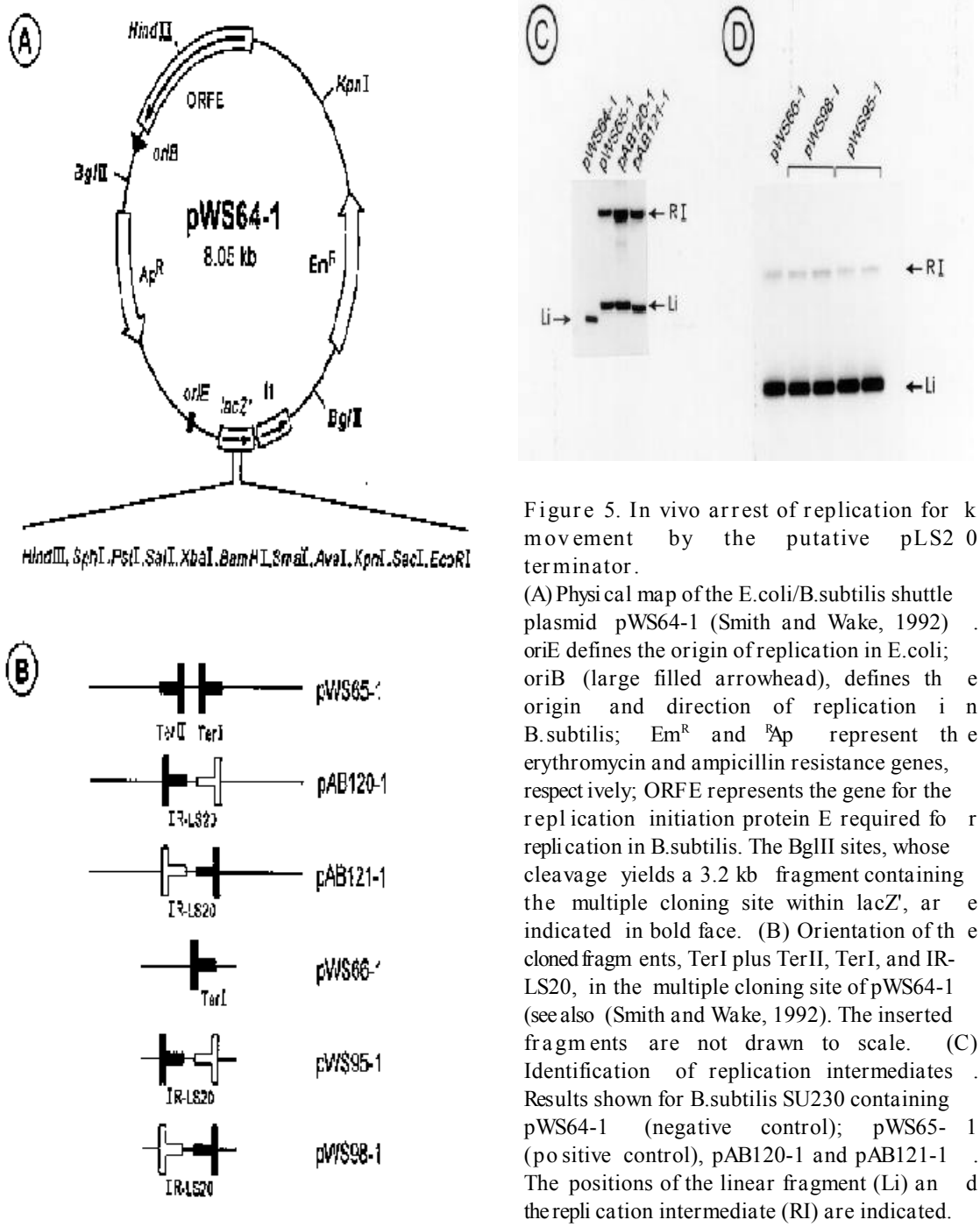

Figure 5. In vivo arrest of replication for k movement by the putative pLS2 0 terminator.

(A) Physi cal map of the E.coli/B.subtilis shuttle plasmid pWS64-1 (Smith and Wake, 1992) . oriE defines the origin of replication in E.coli; oriB (large filled arrowhead), defines th e origin and direction of replication i n B.subtilis; Em^R and ^RAp represent th e erythromycin and ampicillin resistance genes, respect ively; ORFE represents the gene for the repl ication initiation protein E required fo r replic ation in B.subtilis. The BglII sites, whose cleavage yields a 3.2 kb fragment containing the multiple cloning site within lacZ', ar e indicated in bold face. (B) Orientation of th e cloned fragm ents, TerI plus TerII, TerI, and IR-LS20, in the multiple cloning site of pWS64-1 (see also (Smith and Wake, 1992). The inserted fragm ents are not drawn to scale. (C) Identification of replication intermediates . Results shown for B.subtilis SU230 containing pWS64-1 (negative control); pWS65- 1 (po sitive control), pAB120-1 and pAB121-1 . The positions of the linear fragment (Li) an d the repli cation intermediate (RI) are indicated. (D) Identification of replication intermediates. Results shown for B.subtilis SU230 containing pWS66-1 (positive control); pWS98-1 (two independent transformants); pWS95-1 (two independen t transformant s). The positions of the linear fragment (Li) and the replication intermediate (RI) ar e indic ated. The following conditions were used for the experiments shown in (C) and (D): after growth of cultures to mid-exponen tial phase, plasmid DNA was extracted under neutral pH conditions, cut with BglII and analysed after gel electrophoresis by Southern hybridization with pGEM3Zf(+) as a probe.

purpose, plasmids pAB120-1 and pAB121-1 were constructed. The only difference between these pWS64-1 derivatives is the orientation of the putative terminator of pLS20 (Fig. 5B). The sequence of the cloned fragments of pAB120-1 and pAB121-1 is shown in Fig. 3A. In addition to pAB120-1 and pAB121-1, plasmids pWS64-1 and pWS65-1 were used under identical experimental conditions as negative and positive controls (Fig. 5B). pWS64-1, pWS65-1, pAB120-1 and pAB121-1, isolated from E.coli, were introduced into the RTP-overproducing B.subtilis strain SU230 and plasmid DNA was extracted from exponentially growing cultures using the neutral lysis procedure and the DNAs were subsequently digested with BglII. Since all plasmids used contain BglII sites at the fusion points between the pIL253 and pGEM3Zf(+) moieties of pWS64-1, digestion with this enzyme results in two fragments, one comprizing the pIL253 moiety and the other the pGEM3Zf(+) moiety. The BglII digestion mixtures were fractionated by agarose gel electrophoresis and analyzed by Southern hybridization with pGEM3Zf(+) DNA as a probe. Fig. 5C shows the results of this analysis. As expected, pWS64-1 DNA (lane 1) showed only a single fragment (3.2 kb in size), corresponding to the pGEM3Zf(+) moiety of this plasmid. With pWS65-1, pAB120-1 and pAB121-1, the expected corresponding fragment of 3.4 kb (the pGEM3Zf(+) moiety plus the 0.2 kb inserts) was also present, but in these cases an additional band representing a significant amount of retarded DNA was identified. These results clearly indicate that the cloned fragment of pLS20 is able to function in vivo as a replication terminator and, strikingly, that the activity of this terminator is bidirectional.

In the preceding section it was shown that a third RTP dimer was able to bind to this fragment at high RTP dimer levels. Since the experiments on in vivo fork arrest were carried out in an RTP-overproducing host, it was conceivable that binding of a third RTP dimer was required for bidirectional fork arrest. To test this possibility, the experiments on fork arrest were also carried out with the 29 bp IR-LS20 containing plasmids pWS98-1 and pWS95-1, which were shown to bind only two RTP dimers even at high RTP dimer levels (see Fig. 4C). The results, presented in Fig. 5D, show that the 29 bp IR-LS20 sequence is sufficient for fork arrest and that it functions in both orientations. In this experiment pWS66-1 was used as a positive control.

Taken together, the results described in this work prove that the 29 bp imperfect IR region of pLS20 is a functional bidirectional replication terminator which we will call TerLS20.

DISCUSSION

In the present work we describe the identification and characterization of the replication terminator, TerLS20, of the cryptic theta-replicating plasmid pLS20 from B.subtilis. This terminator is located just outside the minimal region required for replication. Two characteristics of TerLS20 are novel: (i) it is the first replication terminator identified on a Gram-positive plasmid; and (ii) it is the first identified replication terminator which is able to arrest fork migration in both directions.

Replication terminators have been shown to be present on various genomes, like chromosomes of Gram-positive and Gram-negative bacteria and some plasmids from Gram-negative bacteria (for reviews see Hill (1992); Yoshikawa and Wake (1993); and Baker (1995). Where analyzed, binding of a specific protein to the terminator was found to be required for fork arrest. In this respect the newly identified terminator of pLS20 is no exception. Moreover, like the other known

terminators, TerLS20 is not required for replication of pLS20. The terminators identified on the E.coli-derived plasmids require binding of the host-encoded Tus protein for their activity. A similar situation was observed for TerLS20, except that the functionally active protein in this case is RTP which has little homology with the Tus protein of E.coli (Bussiere et al., 1995).

Although several similarities exist between TerLS20 and other replication terminators, the B.subtilis chromosomal terminators in particular, there are also striking differences. All other known replication terminators have a polar activity, i.e. they will arrest a replication fork approaching from one direction only. Except for the Gram-negative plasmid P307 (Saadi et al., 1987), which contains only the single terminator TerR1, all other plasmid-located replication terminator regions consist of two opposed terminators. As a consequence, these regions form a replication fork trap, allowing replication forks to enter but not exit this region (Horiuchi and Hidaka, 1988; Hill, 1992). Also the six identified chromosomal terminators from E.coli and B.subtilis are organized to form a replication fork trap. In contrast, TerLS20 is unique in the sense that it does not form a replication fork trap; the 29 bp region constituting this terminator is sufficient to arrest a replication fork approaching from either side and thereby functions as a bidirectional terminator.

An intriguing question is, what causes the bidirectional activity of TerLS20 ? Our results showed similar binding characteristics between the B.subtilis chromosomal terminator TerI and TerLS20 (Fig. 3B) although precise binding affinities have not been measured. This suggests that the RTP-binding sites of TerLS20 are filled similarly to those in TerI. Detailed studies on the interactions between RTP and TerI have shown that the trinucleotide 5'-TAT-3' is part of both RTP-binding sites A and B (Langley et al., 1993). This trinucleotide, which is conserved in most of the B.subtilis terminators, is also present in TerLS20 where it separates the arms of the inverted repeat of TerLS20 (Fig 4A). Conceivably, this triplet is important for cooperative binding of the second RTP dimer via protein-protein interactions. However, in spite of these similarities between TerI and TerLS20, only the latter functions as a bidirectional terminator. It is likely that this important distinction in terminator activity is caused largely by the marked differences in the DNA sequences over one half of the two terminators and the consequent differences in the RTP-DNA interactions and conformation over these halves. This suggestion is based on an extrapolation of the results obtained by Langley et al. (1993). From the latter studies, in which the interactions between RTP and the nucleosides of the B.subtilis chromosomal terminator TerI were analyzed, it was concluded that nucleoside contacts of RTP with the two binding sites A and B, which have distinct sequences, are very different. It was observed that RTP makes more extensive contacts with nucleosides in both DNA strands of the B site than of the A site. Furthermore, RTP appears to bind to the B site first and in doing so assists in filling of the A site (Langley et al., 1993). The A site alone binds RTP poorly (Langley et al., 1993). Characteristic of TerLS20 is its palindromic structure. Each arm of this structure contains a DNA sequence which is highly homologous to the RTP binding site B of TerI. In its functional orientation, the RTP-binding site B of TerI is proximal to the approaching replication fork. Bruand et al. (1991) have demonstrated that arrest of the replication fork occurs specifically in a region of three bp positioned at almost the end of RTP-binding site B (the position of these three nucleotides is indicated in Fig. 4A). Because of the palindromic structure of TerLS20, the corresponding putative arrest sites are positioned at both borders of the terminator. We speculate that this

particular structural organization of TerLS20 is a key factor in the bidirectional activity of this terminator. Recently, important progress has been made towards elucidating the mechanism of fork arrest caused by the binding of RTP to TerI. This includes: (i) the elucidation of the crystal structure of RTP (Bussiere et al., 1995); and (ii) the discovery that B.subtilis RTP bound to TerI is able to impede fork migration in the Gram-negative bacterium E.coli both in vivo (Young and Wake, 1994) and in vitro (Kaul et al., 1994; Sahoo et al., 1995). These new findings will facilitate a detailed mechanistic analysis of the termination process. The RTP-terminator interaction can be used as a model system to study replication termination processes in general.

The terminators of the bidirectionally replicating B.subtilis and E.coli chromosomes are located approximately opposite of the origin, thereby assuring that replication termination is confined to a specific and limited region of the chromosome. In contrast to the situation with these bacterial chromosomes, the plasmid-located replication terminators are located asymmetrically relative to their origins. The terminus region of plasmid R6K is located several kb from its origin. Replication of R6K starts unidirectionally and the replication fork proceeds up to the terminus where it is arrested. A second replicaton fork is then initiated from the same origin and proceeds in the opposite direction and meets the arrested fork at the terminus region (Lovett et al., 1975). The terminators of the unidirectionally-replicating plasmids R1, R100 and P307 are located in close proximity to the plasmid origin of replication (approximately 300 bp apart). Also TerLS20 is located only several hundred basepairs from the replication region of pLS20. It is conceivable that TerLS20, due to replication fork arrest caused by its asymmetrical positition relative to the origin, forces replication of pLS20 to proceed unidirectionally, perhaps in a way similar to R6K. Another function of TerLS20 may be the prevention of overreplication. Recent in vitro experiments carried out with E.coli minichromosomes containing oriC and TerB sites showed that without a functional Ter-Tus complex abnormal modes of replication occur that result in the generation of multimeric DNA. This overreplication is prevented by a functional Ter-Tus complex (Hiasa and Marians, 1994). Deletion of Ter sites from Gram-negative plasmids has been associated with accumulation of plasmid multimers, which also have been suggested to be products of overreplication (Baker, 1995). Especially for low copy number plasmids (and the chromosome) the prevention of multimer formation is important because a dimer would be recognized by the copy number control machinery as two plasmids, yet could not be partitioned into the two daughter cells during division. Moreover, multimer formation can rapidly lead to subpopulations of cells that contain multimers only and, because of the lower amount of segregating units, these cells have a high chance of generating plasmid-free daughter cells (Summers et al., 1993). The importance of avoiding multimer formation is highlighted by the presence of site-specific recombination systems that resolve them. In addition to the Ter sites, the E.coli terminus carries a specific recombination site (dif) which is used for the conversion of chromosome multimers into monomers. Highly homologous sites are also identified on several E.coli theta-replicating plasmids. In this respect, it is worth noting that pLS20 contains a decamer (5'-TTATGTTAAA-3'), 400 bp away from the TerLS20 site, which is identical to part of the above-mentioned resolution sites. So far, we have not tested whether this sequence is involved in plasmid resolution.

MATERIALS AND METHODS

Bacteria and plasmids. Table 1 lists the bacterial strains and plasmids used.

Media. Media for protoplast transformation of B. subtilis were as described by Chang and Cohen (1979). TY medium contained Bacto Tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). TY plates contained in addition 2% agar. Ampicillin, kanamycin and erythromycin were added to final concentrations of 80, 50 and 5 µg/ml, respectively. When regenerating protoplasts were selected for resistance to kanamycin, the concentration was increased to 150 µg/ml.

DNA techniques. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and Southern transfer of DNA to GeneScreen Plus filters (Dupont NEN, Boston, MA, USA) were carried out as described by Sambrook et al. (1989). DNA fragments were isolated from gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, USA). In most cases probe labelling, DNA hybridization, and washing steps were performed using the enhanced chemiluminescence DNA labelling and detection system (Amersham International plc, Amersham, UK). In some cases ^{32}P-labelling was used and hybridization and detection was as described by Smith et al. (1994). Enzymes were obtained from Boehringer, Mannheim (FRG). DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). [^{35}S] dATP (8 µCi/µl; > 1000 Ci/mmole) was obtained from Amersham International. DNA and protein sequences were analyzed using version 6.7 of the PCGene Analysis Program (Intelligenetics Inc., Mountain View, CA, USA). The FASTA algorithm of Lipman and Pearson (1985) was used for protein comparisons in the Atlas of protein and genomic sequences, release September 1993 (MIPS, Martinsried, FRG). PCR was carried out using the Vent DNA polymerase (New England Biolabs, Beverly, USA). The template DNA was denatured for 1 min at 94°C. The primers listed in 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). The sequences of all fragments obtained through PCR were verified.

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).

Band retardation assays. Band retardation studies were carried out using purified Replication Terminator Protein (RTP) and DNA mixtures as described before (Lewis et al., 1989). PvuI- and BstNI-digested pWS66-1, a plasmid which contains the TerI replication terminator of the B.subtilis chromosome, was used a positive control. For pLS20, a 277 bp region, spanning its putative RTP-binding sites, was amplified by PCR using primers -50R and LS16 (Table 2) and pUCHS650 (described below) as template DNA (sequence shown in Fig. 3A). This purified PCR product was mixed with pWS64-1 DNA which had been digested with PvuI + BstNI + BamHI to provide an excess of non-specific DNA and to mimic the positive control situation.

Construction of plasmids. Plasmid pUCHS650 was constructed as follows. The 1.35 kb HindIII fragment containing the pLS20 replication terminator was cloned into the HindIII site of pUC18, resulting in plasmid pUCHH1350. This plasmid was digested with SpeI (restriction site adjacent to the pLS20 terminator) and XbaI (site present in multiple cloning site of pUC18). The resulting 0.65 kb SpeI/XbaI site was subcloned in the XbaI site of pUC18, resulting in pUCHS650.

Table 1. Bacterial strains and plasmids

Strains	Relevant properties	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
SU230	trpC2, Nm^R, $lacI^-$, spac-1 rtp	Smith and Wake, 1992
E.coli		
JM101	thi, Δ(lac-proAB) [F proAB $lacI^q$ lacZΔM15]	Messing, 1979
Plasmids	**Relevant properties**	**Reference**
pUC18	high-copy E.coli vector containing MCS, Ap^R	Yanish-Perron et al., 1985
pUCHH1350	pUC18 with the 1.35 kb HindIII fragment of pLS20 containing TerLS20, Ap^R	This study
pUCHS650	pUC18 with 0.65 kb TerLS20 containing SpeI/HindIII fragment of pLS20, Ap^R	This study
pWS64-1	E.coli/B.subtilis shuttle plasmid based on pIL253 and pGEM3Zf(+), Ap^R, Em^R	Smith and Wake, 1992
pWS65-1	pWS64-1 with a 0.2 kb fragment containing the B.subtilis 168 chromosomal TerI and TerII	Smith and Wake, 1992
pWS66-1	pWS64-1 with a 53 bp fragment containing the B.subtilis 168 chromosomal terminator TerI	Smith and Wake, 1992
pAB120-1	pWS64-1 with 0.2 kb fragment of pLS20 containing TerLS20	This study
pAB121-1	pWS64-1 with 0.2 kb fragment of pLS20 containing TerLS20 (inverse orientation compared to pAB120-1)	This study
pWS95-1	pWS64-1 with 29 bp TerLS20 fragment	This study
pWS98-1	pWS64-1 with 29 bp TerLS20 fragment (inverse orientation compared to pWS95-1)	This study

Abbreviations used: MCS, multiple cloning site, Ap^R, ampicillin resistance, Em^R, erythromycin

Table 2. Primers used

Name	Sequence (5'-3') *
TER1	GATCGAGCTCGTCGA CTAACGTATACTGTTATTCCC
LS20-16	ATACTGTTATTCCCTTTGGCC
-50R	GCTCGTATGTTGTGTGG
LS20-95A	CTA GAACATTAAGCACTATGTACTAAATGTT
LS20-95B	CTA GAACATTTAGTACATAGTGCTTAATGTT
LS20-98A	AATTC GAACATTAAGCACTATGTACTAAATGTTC G
LS20-98B	GATCC GAACATTTAGTACATAGTGCTTAATGTTC G

*: Underlined bases represent extensions to generate appropriate restriction

Plasmids pAB120-1, pAB121-1, pWS98-1 and pWS95-1 are derivatives of pWS64-1 (Smith and Wake, 1992). pWS1201-1 and pAB121-1 were constructed as follows. A 0.30 kb fragment spanning the pLS20 replication terminator was amplified by PCR using pUCHS650 as template DNA and the primers TER1 and -50R (Table 2; sequence in Fig. 3A). Consequently, the amplified PCR products contain a unique SacI and SalI site at one terminus (an extension in primer TER1) and unique EcoRI and BamHI sites at the other terminus (due to amplification of part of the multiple cloning site of pUC18). The PCR products were divided in two aliquots, one of which was digested with SacI and BamHI, and the other with SalI and EcoRI. Subsequently, the DNA fragments were ligated into the SacI/BamHI and SalI/EcoRI sites of pWS64-1. The ligation mixtures were used to transform E.coli cells. Using restriction analyses on plasmid DNA isolated from transformants, plasmids containing the replication terminator of pLS20 in either orientation were identified. The integrity of the inserts was confirmed by sequence analysis. pWS95-1 was constructed by annealing primer set LS20-95A and LS20-95B and ligating to pWS64-1 cut with XbaI. pWS98-1 was constructed by annealing primer set LS20-98A and LS2098B and ligating to pWS64-1 cut with EcoRI and BamHI. The integrity of the inserts was confirmed by sequencing.

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REFERENCES

Ahn, K.S., Malo, M.S., Smith, M.T., and Wake, R.G. (1993). Autoregulation of the gene encoding the replication terminator protein of Bacillus subtilis. Gene 132: 7-13.

Baker, T.A. (1995). Replication arrest. Cell 80: 521-524.

Bird, R.E., Louarn, J., Martuscelli, J., and Caro, L. (1972). Origin and sequence of chromosome replication in Escherichia coli. J. Mol. Biol. 70: 549-566.

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

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

Bruand, C., Ehrlich, S.D., and Jannière, L. (1991). Unidirectional theta replication of the structurally stable Enterococcus faecalis plasmid pAM β1. EMBO J. 10: 2171-2177.

Bussiere, D.E., Bastia, D., and White, S.W. (1995). Crystal structure of the replication terminator protein from B.subtilis at 2.6 Å. Cell 80: 651-660.

Carrigan, C.M., Haarsma, J.A., Smith, M.T., and Wake, R.G. (1987). Sequence features of the replication terminus of the Bacillus subtilis chromosome. Nucleic Acids Res. 15: 8501-8509.

Chang, S. and Cohen, S.N. (1979). High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA. Mol. Gen. Genet. 168: 111-115.

Franks, A.H., Griffiths, A.A., and Wake, R.G. (1995). Identification and characterization of new DNA replication terminators in Bacillus subtilis. Mol. Microbiol. In Press

Freid, M. and Crothers, D. (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. 9: 6505-6525.

Hiasa, H. and Marians, K.J. (1994). Tus prevents overreplication of oriC plasmid DNA. J. Biol. Chem. 269: 26959-26968.

Hill, T.M., Pelletier, A.J., Tecklenburg, M., and Kuempel, P.L. (1988). Identification of the DNA sequence from the E.coli terminus region that halts replication forks. Cell 55: 459-466.

Hill, T.M., Tecklenberg, M., Pelletier, A.J., and Kuempel, P.L. (1989). tus, the trans-acting gene required for termination of DNA replication in Escherichia coli, encodes a DNA-binding protein. Proc. Natl. Acad. Sci. USA 86: 1593-1597.

Hill, T.M. (1992). Arrest of bacterial DNA replication. Annu. Rev. Microbiol. 46: 603-633.

Horiuchi, T. and Hidaka, M. (1988). Core sequence of two separable terminus sites of the R6K plasmid that exhibit polar inhibition of replication is a 20 bp inverted repeat. Cell 54: 515-523.

Iismaa, T.P., Smith, M.T., and Wake, R.G. (1984). Physical map of the Bacillus subtilis replication terminus region: its confirmation, extension and genetic orientation. Gene 32: 171-180.

Kaul, S., Mohanty, B.K., Sahoo, T., Patel, I., Khan, S., and Bastia, D. (1994). The replication terminator protein of the Gram-positive bacterium Bacillus subtilis functions as a polar contrahelicase in Gram-negative Escherichia coli. Proc. Natl. Acad. Sci. USA 91: 11143-11147.

Koehler, T.M. and Thorne, C.B. (1987). Bacillus subtilis (natto) plasmid pLS20 mediates interspecies plasmid transfer. J. Bacteriol. 169: 5271-5278.

Kuempel, P.L., Duerr, S.A., and Seeley, N.R. (1977). Terminus region of the chromosome in Escherichia coli inhibits replication forks. Proc. Natl. Acad. Sci. USA 74: 3927-3931.

Kuempel, P.L., Duerr, S.A., and Maglothin, P.D. (1978). Chromosome replication in an Escherichia coli dnaA mutant integratively suppressed by prophage P2. J. Bacteriol. 134: 902-912.

Langley, D.B., Smith, M.T., Lewis, P.J., and Wake, R.G. (1993). Protein-Nucleoside Contacts in the Interaction Between the Replication Terminator Protein of Bacillus-subtilis and the DNA Terminator. Mol. Microbiol. 10: 771-779.

Lewis, P.J., Smith, M.T., and Wake, R.G. (1989). A protein involved in termination of

chromosome replication in Bacillus subtilis binds specifically to the terC site. J. Bacteriol. 171: 3564-3567.

Lewis, P.J., Ralston, G.B., Christopherson, R.I., and Wake, R.G. (1990). Identification of the replication terminator protein binding sites in the terminus region of the Bacillus subtilis chromosome and stoichiometry of the binding. J. Mol. Biol. 214: 73-84.

Lewis, P.J. and Wake, R.G. (1989). DNA and protein sequence conservation at the replication terminus in Bacillus subtilis 168 and W23. J. Bacteriol. 171: 1402-1408.

Louarn, J., Patte, J., and Louarn, J.-M. (1977). Evidence for a fixed termination site of chromosome replication in Escherichia coli K12. J. Mol. Biol. 115: 295-314.

Louarn, J., Patte, J., and Louarn, J.-M. (1979). Map position of the replication terminus on the Escherichia coli chromosome. Mol. Gen. Genet. 172: 7-11.

Lovett, M.A., Sparks, R.B., and Helinski, D.R. 1975). Bidirectional replication of plasmid R6K DNA in Escherichia coli; replication and position of single-strand break in relaxed complex. Proc. Natl. Acad. Sci. USA 72: 2905-2909.

Masters, M. and Broda, P. (1971). Evidence for the bidirectional replication of the Escherichia coli chromosome. Nature 232: 137-140.

Messing, J. (1979). A multipurpose cloning system based on the single stranded DNA bacteriophage M13. Recombinant DNA technical Bullitin, NIH publication 79-99.2: 43-48.

Miyazaki, C., Kawai, Y., Ohtsubo, H., and Ohtsubo, E. (1988). Unidirectional replication of plasmid R100. J. Mol. Biol. 204: 331-343.

Mueller, J.P., Bukusoglu, G., and Sonenshein, A.L. (1992). Transcriptional regulation of Bacillus subtilis glucose starvation-inducible genes: control of gsiA by the ComP-ComA signal transduction system. J. Bacteriol. 174: 4361-4373.

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

Perego, M., Hanstein, C., Welsh, K.M., Djavakhishvili, T., Glaser, P., and Hoch, J.A. (1994). Multiple protein aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in Bacillus subtilis. Cell 79: 1047-1055.

Roecklein, B.A. and Kuempel, P.L. (1992). In vivo characterization of tus gene expression in Escherichia coli. Mol. Microbiol. 6: 1655-1661.

Saadi, S., Maas, W.K., Hill, D.F., and Berquist, P.L. (1987). Nucleotide sequence analysis of RepFIC, a basic replicon present in IncFI plasmids P307 and F, and its relation to the RepA replicon of IncFII plasmids. J. Bacteriol. 169: 1836-1846.

Sahoo, T., Mohanty, B.K., Patel, I., and Bastia, D. (1995). Termination of DNA replication in vitro: requirements for stereospecific interaction between two dimers of the replication terminator protein of Bacillus subtilis and with the terminator site to elicit polar contrahelicase and fork impedence. EMBO J. 14: 619-628.

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

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Sista, P.R., Mukherjee, S., Patel, P., Khatri, G.S., and Bastia, D. (1989). A host-encoded DNA-binding protein promotes termination of plasmid replication at a sequence-specific replication terminus. Proc. Natl. Acad. Sci. USA 86: 3026-3030.

Smith, M.T., Langley, D.B., Young, P.A., and Wake, R.G. (1994). The minimal sequence needed to define a functional DNA terminator in Bacillus subtilis. J. Mol. Biol. 241: 335-340.

Smith, M.T. and Wake, R.G. (1992). Definition and polarity of action of DNA replication terminators in Bacillus subtilis. J. Mol. Biol. 227: 648-657.

Summers, D.K., Beton, C.W.H., and Withers, H.L. (1993). Multicopy plasmid instability: the dimer catastrophe

hypothesis. Mol. Microbiol. 8: 1031-1038.

Tanaka, T. and Koshikawa, T. (1977). Isolation and characterization of four types of plasmids from Bacillus subtilis (natto). J. Bacteriol. 131: 699-701.

Weiss, A.S. and Wake, R.G. (1983). Restriction map of DNA spanning the replication terminus of the Bacillus subtilis chromosome. J. Mol. Biol. 171: 119-137.

Yanish-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

Yoshikawa, H. and Wake, R.G. (1993). Initiation and termination of chromosome replication. In Bacillus subtilis and other Gram-positive bacteria: biochemistry physiology, and molecular genetics. Sonenshein, A.L., Hoch, J.A., and Losick, R. (eds.) Washington D.C. American Society for Microbiology, pp. 507-528.

Young, P.A. and Wake, R.G. (1994). The Bacillus subtilis replication terminator system functions in Escherichia coli. J. Mol. Biol. 240: 275-280.