The EMBO Journal vol.10 no.8 pp.2171 - 2177, 1991

Unidirectional theta replication of the structurally stable *Enterococcus faecalis* plasmid pAMβ1

Claude Bruand, S.Dusko Ehrlich and Laurent Jannière

Laboratoire de Génétique Microbienne, Institut de Biotechnologie, INRA-Domaine de Vilvert, 78352 Jouy en Josas Cedex, France

Communicated by S.D.Ehrlich

Numerous bacterial replicons remain poorly characterized due to difficulties in localization of the replication origin. We have circumvented this problem in the characterization and fine mapping of the origin of plasmid pAMβ1 by exploiting the *Bacillus subtilis* termination signal, *terC*. In *terC*-containing derivatives, θ-form molecules with two invariant endpoints accumulate. The endpoints, which correspond to plasmid origin and *ter*C, were mapped with single-nucleotide precision. Analysis of the replication intermediates of wild-type molecules by two-dimensional gel electrophoresis confirmed the location of the plasmid origin. Our results demonstrate that pAMβ1 replication proceeds unidirectionally by a theta mechanism. This work confirms the use of termination signals to localize origins, suggests that termination in *B.subtilis* occurs by a mechanism similar to that of *Escherichia coli* and establishes that in addition to rolling circle replicating plasmids, Gram positive bacteria harbour plasmids which replicate by a theta mechanism.

Key words: Gram positive bacteria/plasmid family/ replication origin/replication terminus

Introduction

Numerous small plasmids (< 10 kb) have been isolated from Gram positive bacteria. Comparison of their replication regions has indicated that they belong to four different groups (Gruss and Ehrlich, 1989). Studies of representative plasmids from three of these groups, pC194, pE194 and pT181, have shown that they replicate by a rolling circle mechanism (Sozhamannan *et al.*, 1990; for reviews see Gruss and Ehrlich, 1989; Novick, 1989) similar to that of ssDNA phages (reviewed by Baas and Jansz, 1988; Model and Russel, 1988). Less extensive studies of several other small plasmids led to the hypothesis that all small plasmids from Gram positive bacteria replicate as rolling circles. These plasmids are referred to as ssDNA plasmids (Gruss and Ehrlich, 1989), emphasizing their property to generate circular single-stranded DNA replication intermediates (te Riele *et al.*, 1986a,b; Gros *et al.*, 1987, 1989), and form a widespread family.

Numerous large (> 10 kb) plasmids have also been detected in Gram positive bacteria. Several observations suggest that they do not replicate by a rolling circle mechanism: (i) small derivatives of the large plasmids pAMβ1 (26.5 kb, Clewell *et al.*, 1974), pTB19 (26 kb, the *repA* region, Imanaka *et al.*, 1981) and pIP404 (10.2 kb, Brefort *et al.*, 1977) do not accumulate single-stranded DNA during replication (Garnier and Cole, 1988; Jannière *et al.*, 1990); (ii) replication regions of these plasmids share no sequence homology with the highly conserved (+) and (−) origins of the rolling circle type plasmids (Garnier and Cole, 1988; Jannière *et al.*, 1990; Swinfield *et al.*, 1990); (iii) organization of the replication regions of pTB19 (the *repA* region) and pIP404 resembles that of theta-replicating plasmids from Gram negative bacteria (Imanaka *et al.*, 1986; Garnier and Cole, 1988).

In this report we show that the large *Enterococcus faecalis* plasmid pAMβ1 undergoes unidirectional theta replication in *B.subtilis* cells. First, analysis of a small derivative by two-dimensional (2-D) gel electrophoresis (Brewer and Fangman, 1987; Brewer *et al.*, 1988) revealed θ-shaped replication intermediates in which replication initiated at a fixed origin and progressed in only one direction. Second, insertion of the replication terminus of the *B.subtilis* chromosome (Weiss and Wake, 1984; Smith and Wake, 1988) into another small derivative plasmid led to accumulation of partially supercoiled θ-shaped molecules in which one of the branching sites mapped at the replication origin and another at the terminus, as deduced from electron microscopy analysis. In addition, using the accumulated θ-shaped molecules we determined at the nucleotide level the sites of initiation and arrest of the leading and lagging DNA strand synthesis at the origin and terminus of replication. These results indicate the existence of two plasmid families in Gram positive bacteria, using rolling circle or theta replication respectively. Since plasmids belonging to the former family are structurally less stable than those belonging to the latter (Jannière *et al.*, 1990; for reviews see Ehrlich *et al.*, 1986; Gruss and Ehrlich, 1989), a relationship between the mode of genome replication and its structural stability might exist.

Results

Electrophoretic analysis of pAMβ1 replication

Theta replication generates intermediates which have a typical shape, often called replication bubble. To determine whether pAMβ1 replication generates such intermediates we analysed a 5.7 kb derivative plasmid, pIL206 (Simon and Chopin, 1988), by 2-D agarose gel electrophoresis (Brewer and Fangman 1987; Brewer *et al.*, 1988). This plasmid comprises the pAMβ1 replication region (the large *Hpa*I−*Eco*RI segment, Swinfield *et al.*, 1990), the *Em* resistance gene of pAMβ1 and carries a single *Nhe*I restriction site (Figure 1). Upon *Nhe*I cleavage of total DNA prepared from a *B.subtilis* strain harbouring pIL206 and 2-D gel electrophoresis, an arc which starts at the position of linear monomers (5.7 kb) and stretches towards circular dimers was observed (Figure 2, top). It indicates the existence of bubble-shaped replication intermediates and suggests that pIL206 replicates by a theta-type mechanism.

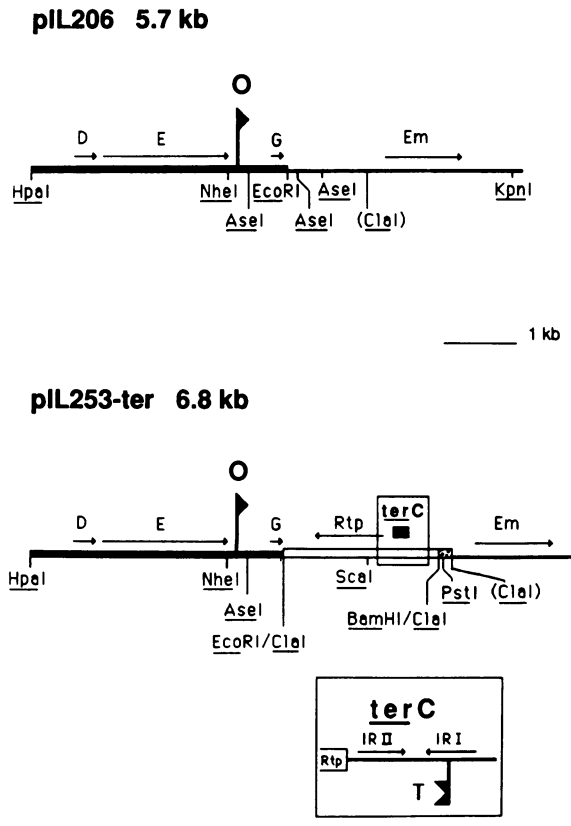

Fig. 1. Structure of pIL206 and pIL253-ter. The drawing represents plasmids linearized at the unique *Hpa*I site. Thick and thin lines stand for the replication region and other pAMβ1 sequences, including the *Em* gene, respectively. Open and dashed boxes represent the terminus of the *B.subtilis* chromosome and the polylinker region of pIL253, respectively. Key restriction sites are indicated. Different ORFs are represented by arrows. O indicates the location of the pAMβ1 origin and the black triangle the direction of replication. The replication pause site of the *B.subtilis* terminus, *terC*, is indicated by a black box and expanded at the bottom. Inverted repeats (IR) are indicated by arrows, the replication pause site (T) by a flag.

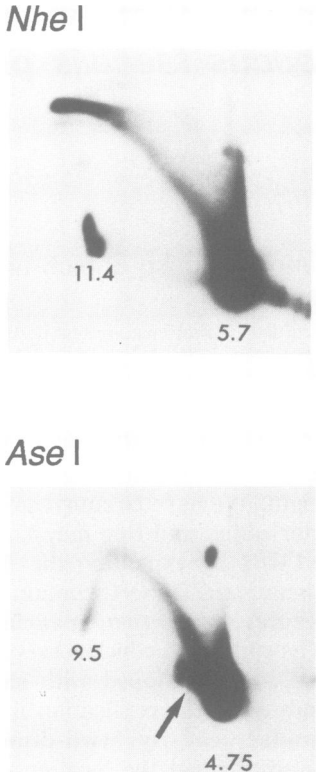

Fig. 2. 2-D gel electrophoresis of pIL206 replication intermediates. The total DNA extracted from *B.subtilis* strain MI112 harbouring pIL206 was restricted with *Nhe*I or *Ase*I, the segments were separated by 2-D gel electrophoresis, transferred on a nitrocellulose filter and hybridized with the related pIL253 DNA labelled by nick translation. The arcs observed after *Nhe*I and *Ase*I restriction indicate the existence of bubble-shaped and Y-shaped replication intermediates, respectively. The arrow in the *Ase*I panel pin-points in the arc an accumulation of nascent replication intermediates clearly visible on a less exposed film (not shown). The numbers refer to segment sizes (in kb). The first dimension was from the left to the right, the second from the top to the bottom.

An additional hybridization signal close to the region of linear dimers (11.4 kb) indicates the presence of large Y-shaped molecules, presumably generated from intermediates in which replication has progressed past the cleavage site.

The arc could result from either unidirectional replication initiated close to the *Nhe*I site and progressing away from it, or bidirectional replication initiated far from the *Nhe*I site (e.g. near the *Hpa*I site, Figure 1). To distinguish between these alternatives we cleaved pIL206 with *Ase*I and determined the pattern given by the largest segment (*Ase*I cleavage generates three segments, one of 4.75 kb and two of <1 kb, see Figure 1). The resulting arc stretched between the positions of linear monomers (4.75 kb) and dimers (9.5 kb), which indicates the presence of Y-shaped molecules (Figure 2, bottom). We conclude that pIL206 replicates unidirectionally from an origin located close to the *Nhe*I site, since the bidirectional replication initiated far from this site would generate large *Ase*I segments carrying bubbles. Moreover, replication must have been initiated right of the *Nhe*I site and progressed rightwards (towards the *Em* gene, cf. Figure 1), since if it were initiated left of this site and progressed leftwards the large *Ase*I segment would also carry bubbles. The replication origin of pIL206 is therefore localized between the *Nhe*I site and the *Eco*RI site which delimits the pAMβ1 replication region (Figure 1).

The spot in the arc of the *Ase*I profile (pin-pointed by an arrow in Figure 2 and more visible on a less exposed film) could be due to an accumulation of large *Ase*I segments carrying a short fork at one end. Such an accumulation might result from cleavage of nascent intermediates in which the replication fork initiated close to the *Nhe*I site was localized between the proximal and the distal *Ase*I sites (Figure 1). Further progression of the fork, past the distal *Ase*I site, would lead to the appearance of segments carrying a short fork at one end and a progressively longer fork at the other, giving an arc such as that displayed in the bottom panel of Figure 2.

Taken together, the above results indicate that pAMβ1 undergoes unidirectional theta replication initiated between the *Nhe*I and the closest *Ase*I site and progressing towards the *Ase*I site.

Electron microscopy analysis of pAMβ1 replication

To confirm the type of pAMβ1 replication and the localization of its replication origin we attempted to isolate replication intermediates of plasmid pIL253-ter. This plasmid (Figure 1), related to pIL206, comprises the replication region and the erythromycin resistance gene of pAMβ1 as well as a segment of *B.subtilis* DNA encoding the signal

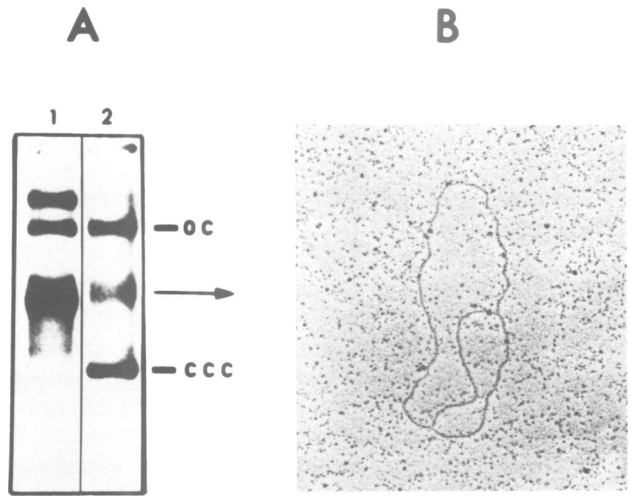

Fig. 3. Gel electrophoresis and electron microscopy of pIL253-ter replication intermediates. (**A**) Gel electrophoresis. DNA prepared from *B.subtilis* cells harbouring pIL253-ter was hybridized with pIL253 probe. Lane 1. Intermediate density DNA purified by CsCl-ethidium bromide centrifugation. Lane 2. Total DNA. Positions of covalently closed (ccc) and open (oc) circles are shown. The arrow indicates the band of the intermediate mobility. (**B**) Electron microscopy. Intermediate density DNA was relaxed and prepared for electron microscopy as indicated in Materials and methods. More than 50% of the molecules carried a bubble.

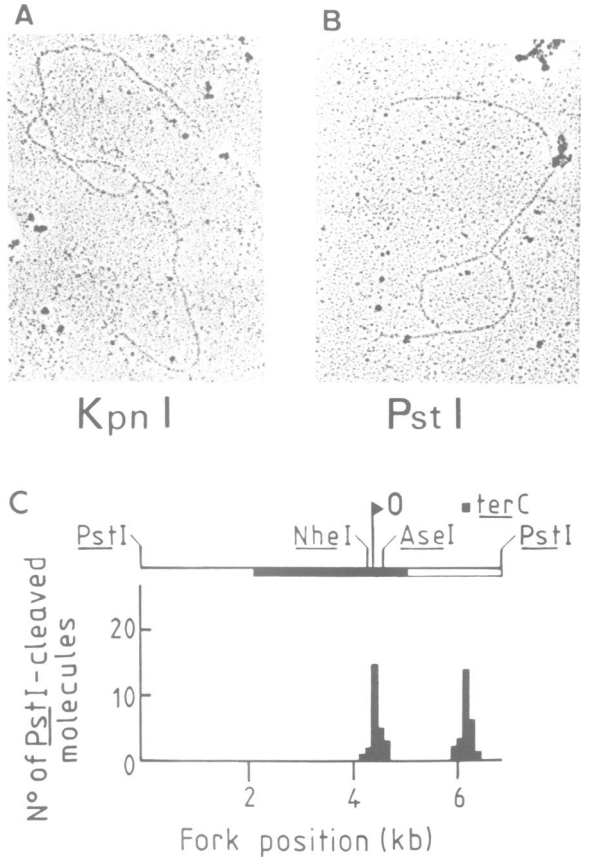

Fig. 4. Localization of the forks present on pIL253-ter replication intermediates. Accumulated replication intermediates enriched by CsCl-gradient density were linearized with *Kpn*I (**A**) or *Pst*I (**B**) and visualized by electron microscopy. Position of the forks was determined by measuring the lengths of arms and bubbles in 26 molecules cleaved by *Pst*I (**C, bottom**). Fork positions are aligned with a schematic representation of pIL253-ter (**C, top**). Symbols are as indicated in Figure 1.

which terminates chromosomal replication (*terC*) and the cognate replication terminator protein (RTP, Carrigan *et al.*, 1987). ColE1-derived plasmids carrying equivalent *E.coli* signals accumulate θ-form replication intermediates, in which one fork maps at the origin and the other at the terminus (Horiuchi *et al.*, 1987; Pelletier *et al.*, 1989).

To search for accumulation of pIL253-ter replication intermediates, the total DNA of a strain harbouring this plasmid (a *recE4* strain was used to avoid recombination between homologous plasmid and chromosome regions) was extracted, separated on an agarose gel, transferred onto a nitrocellulose filter and hybridized with labelled pIL253 DNA (Figure 3A, lane 2). Three bands of similar intensities were observed, at positions of covalently closed and open circles, and at an intermediary position. Centrifugation of plasmid DNA in CsCl−ethidium bromide density gradients also revealed three bands, two at positions of supercoiled and relaxed molecules and the third at an intermediary position (not shown). Agarose gel electrophoresis indicated that the last band consisted mainly of the plasmid form having the intermediate mobility (Figure 3A, lane 1) and the electron microscopy revealed that it contained plasmid molecules carrying a replication bubble (Figure 3B). This result shows that pIL253-ter accumulates partially supercoiled bubble-shaped molecules.

If the accumulated bubble-shaped molecules are replication intermediates generated by a unidirectional theta-type replication, one of the forks should be located at the pAMβ1 origin, another at the *B.subtilis* terminus. To determine fork positions, the intermediates (enriched by density gradients) were treated with enzymes which cleave the plasmid only once (*Kpn*I or *Pst*I were used, Figure 1) and analysed by electron microscopy (Figure 4A and B). Measurements of 26 *Pst*I treated molecules established that the size of the bubble was 1700 ± 120 bp and that the two branches map at 660 ± 130 bp and 4300 ± 100 bp from the site of cleavage (Figure 4C, bottom). One of the two possible positions fits well the known locations of the pAMβ1 origin (as determined above by 2-D gel electrophoresis) and the replication pause site *terC* of the *B.subtilis* terminus (Figure 4C, top). Analysis of three *Kpn*I cleaved molecules supported this interpretation (not shown). We conclude from these results that the terminus of *B.subtilis* chromosome arrests pAMβ1 replication at *terC* and that pAMβ1 undergoes unidirectional theta replication initiated in the small *Nhe*I−*Ase*I segment and progressing towards the *Ase*I site.

Precise mapping of the replication origin and terminus in pIL253-ter

To determine the precise location of the replication origin and terminus in pIL253-ter, we mapped the 5′ and 3′ ends of the newly synthesized strands present in the accumulated pIL253-ter replication intermediates. The enriched intermediates, treated with *Pst*I (which cuts outside the replication bubble, Figure 4B), were heated to 80°C to extrude, by branch migration, the newly synthesized DNA strands from the bubble (Horiuchi and Hidaka, 1988). The resulting DNA preparation contained two double-stranded segments: a 1.7 kb segment corresponding to the extruded newly synthesized strands and a 6.8 kb segment corresponding to the linear plasmid identical to a non-replicating form (not shown).

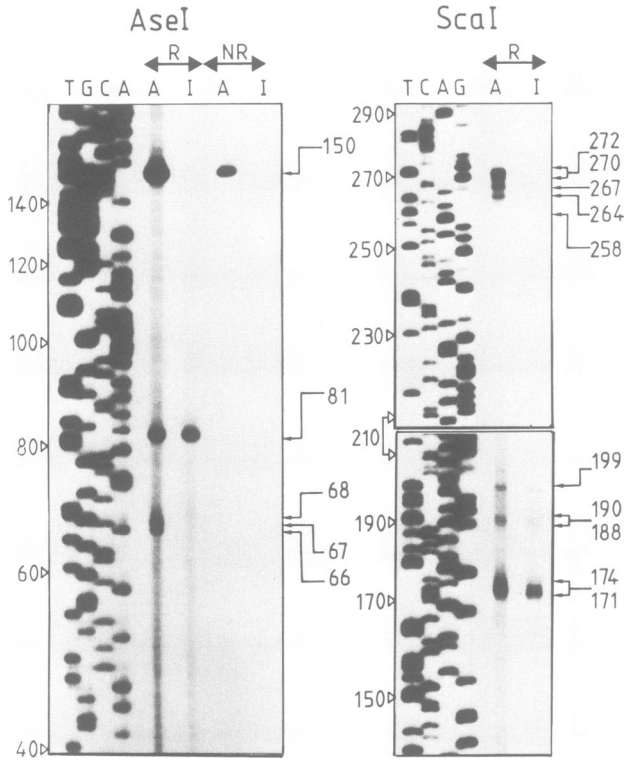

Fig. 5. Mapping of 5′ and 3′ ends of the newly synthesized strands in pIL253-ter replication intermediates. Plasmid DNA was purified by CsCl–ethidium bromide density gradient centrifugation, cleaved with *PstI*, heated to 80°C and labelled as indicated in the text and in Materials and methods. Cleavage with *AseI* and *ScaI* was used for mapping in the origin and terminus region, respectively. R and NR denote lanes containing enriched replication intermediates and non-replicating plasmid DNA, respectively; I and A the lanes allowing precise mapping of the 5′ and 3′ ends, respectively. A M13 sequencing ladder was used as a size marker. The numbers refer to segment length in base pairs.

To map the 5′ ends of the extruded segment, DNA molecules prepared as described above were labelled by a phosphatase–polynucleotide kinase treatment, cleaved either with *AseI*, which cuts close to the origin, or with *ScaI*, which cuts close to the terminus (both enzymes cut in the replication bubble, see Figure 1), and analysed on a sequencing gel (Figure 5, lanes R/I; only the short DNA segments are displayed). In a control experiment, non-replicating plasmid forms, purified by CsCl gradients and cleaved with *AseI*, were used instead of replication intermediates (Figure 5, lane NR/I). The size of the labelled segments detected on the gel and specific for replication intermediates (compare lanes R and NR) should correspond to the distance between the 5′ ends of the extruded segment and the site of cleavage. A single segment, of 81 bp, was generated by *AseI* whereas several segments were generated by *ScaI*, the most prominent being those of 171–174 bp. This indicates the existence of a single initiation site of the leading strand synthesis at the origin and of several initiation sites of the lagging strand synthesis at the terminus. The positions of these sites are represented in Figure 6.

To map the 3′ ends of newly synthesized strands, the segments resulting from *PstI* cleavage and branch migration were cut with *AseI* or *ScaI*, dephosphorylated, labelled by kinase treatment and analysed on a sequencing gel. Two types of labelled segments should specifically derive from replication intermediates molecules: (i) those corresponding to the distance between the 5′ ends of the extruded segment and the cleavage site and which must match the segments detected by the procedure described above and (ii) those corresponding to the distance between the 3′ ends of the extruded segment and the cleavage site. Several short segments were observed upon *AseI* cleavage (Figure 5, lane R/A). One, of 81 bp, matches the segment detected above and thus corresponds to a 5′ end of the extruded segment. Another, of 150 bp, results from *AseI* cleavage of the non-replicating molecules, as judged from the control experiment (Figure 5, lane NR/A). The three remaining segments (66–68 bp) result from 3′ ends of the extruded segment at the origin. Similar analysis of the segments generated by *ScaI* cleavage (all the fragments displayed are specific for replication intermediates, not shown) identified several 3′ ends at the terminus, prominently at the position mapping 270–272 bp from the cleavage site. These data indicate the existence of several arrest sites of the lagging and leading strands synthesis at the origin and terminus of replication, respectively. Positions of the arrest sites are represented in Figure 6.

Discussion

pAMβ1, a large (26.5 kb), broad host range plasmid isolated from the Gram positive bacterium *E.faecalis* (Clewell *et al.*, 1974), replicates by a unidirectional theta mechanism in *B.subtilis* cells, as deduced from the detection and analysis of θ-shaped replication intermediates by 2-D gel electrophoresis (Figure 2). Its origin is located downstream from the gene encoding the replication protein RepE and its replication progresses in the direction of transcription of this gene (Figure 1). The terminus of the *B.subtilis* chromosome arrests pAMβ1 replication *in vivo*, leading to accumulation of partially supercoiled θ-shaped molecules in which one fork maps at the pAMβ1 origin, another at the replication pause site *terC*, as shown by electron microscopy analysis (Figures 3 and 4). These molecules were purified by CsCl gradients and used to map precisely the sites of initiation and arrest of the leading and lagging strand DNA synthesis.

At the origin, leading strand synthesis was initiated at a single site (Figures 5 and 6). The only remarkable features in the vicinity of this site are two inverted repeats (Figure 6), flanked by a nearly perfect 15 bp long direct repeat. Structures frequently observed in plasmid and chromosomal origins active in Gram negative bacteria and in the *B.subtilis* chromosomal origin, such as short direct repeats, dnaA boxes and AT-rich regions (Bramhill and Kornberg, 1988; Ogasawara *et al.*, 1990) are not present close to the initiation site. This suggests that pAMβ1 origin might function in a different way. This function is compatible with numerous organisms, since pAMβ1 is a very broad host range replicon (reviewed by Horaud *et al.*, 1985).

Three arrest sites of lagging strand synthesis were observed in the origin (Figures 5 and 6). One cannot rigorously exclude the possibility that this heterogeneity is a consequence of degradation of the newly synthesized strands during DNA preparation, but it is thought unlikely, since heterogeneity was not observed at the initiation site of leading strand synthesis, and no ladder of bands of

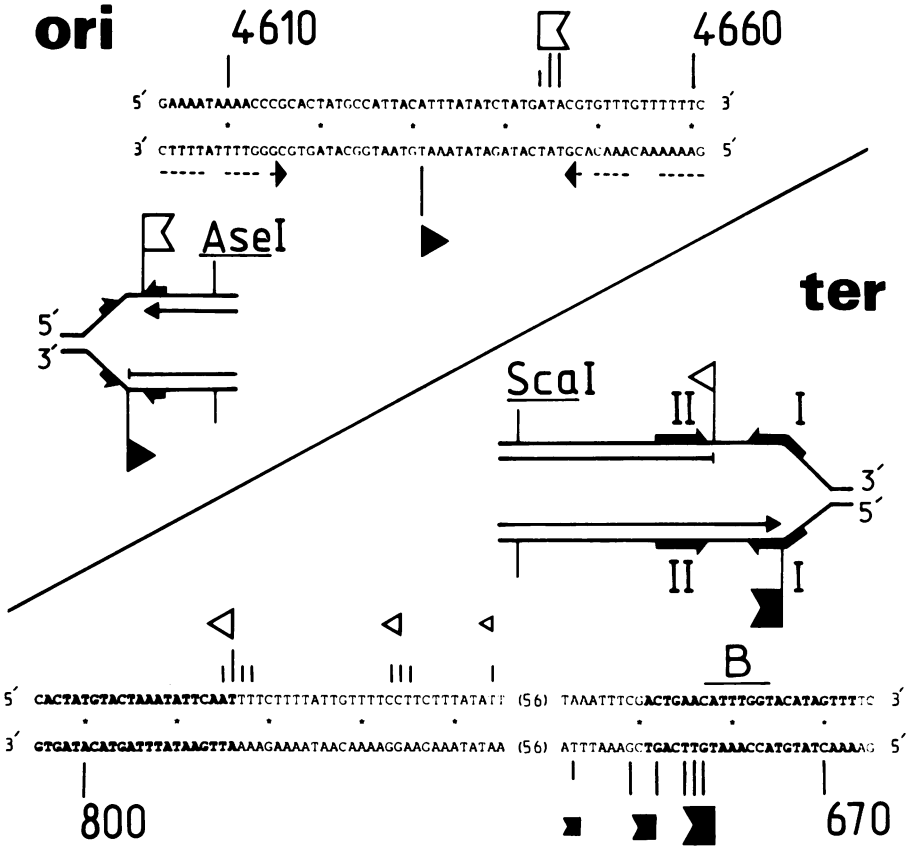

Fig. 6. Location of the initiation and arrest sites of the leading and lagging strand synthesis at the origin and terminus. Replication forks at the origin (ori) and terminus (ter) are schematically presented in the middle of the figure, the relevant nucleotide sequences are displayed at the top and the bottom. Triangles and flags denote initiation and arrest sites, respectively, open and closed symbols refer to leading and lagging strand, respectively. Major and minor sites are represented by large and small symbols, respectively. Relevant restriction sites are indicated. Heavy and dashed arrows represent inverted repeats. The RTP binding site B in IR I is indicated. Bold face nucleotides in the terminus are homologous to the *E.coli* terminators (Hill *et al.*, 1988).

decreasing intensity was noticed below the main band. The synthesis of lagging strand is arrested before reaching the site of initiation of the leading strand. This results in a 13–16 bp region of single-stranded DNA at the replication origin. Single-stranded regions were also detected at origins of plasmids pBR322 and R100 during *in vitro* replication, except that the arrest sites were situated beyond rather than in front of the initiation sites (Dasgupta *et al.*, 1987; Miyazaki *et al.*, 1988). The arrest sites of the lagging strand synthesis in the pAMβ1 origin are not in the vicinity of a sequence homologous to previously described terminators (Hill *et al.*, 1988; Horiuchi and Hidaka, 1988; Carrigan *et al.*, 1987) which suggests that termination occurs by a different mechanism. It was proposed that transcription might arrest replication (Brewer and Fangman, 1988; Linskens and Huberman, 1988) and it is interesting to notice that the arrest sites in the pAMβ1 origin are localized 43–45 nucleotides downstream from the *repE* gene.

At the terminus, leading strand synthesis was arrested (or severely impeded) at three main and three minor sites within IR I (Figures 5 and 6). Before being arrested, the synthesis passes through the *rtp* gene and IR II, which fits well the observations reported for the *B.subtilis* chromosome when the clockwise moving fork is arrested at *terC* (Weiss and Wake, 1984; Carrigan *et al.*, 1987; Williams and Wake, 1989). This suggests that the arrest of plasmid and chromosomal forks is governed by the same mechanism.

Recently, four binding sites for RTP protein have been detected in the *ter*C region (Lewis et al., 1990), probably corresponding to an 8 bp sequence present twice within IR I and twice within IR II. Our data show that the main arrests of the leading strand synthesis take place one and two nucleotides upstream of and one nucleotide within the binding site B of IR I (Figure 6). This is the first binding site in the active orientation to be encountered by the replication fork, according to the model proposed by Lewis *et al.* (1990). The position of the arrest sites suggests that the RTP protein is bound to the DNA when the polymerase approaches IR I and that DNA synthesis continues until it reaches the sequence bound by RTP. In *E.coli*, the arrest of the leading strand synthesis at the terminator T2 might be governed by a similar mechanism, since: (i) this terminator carries the binding site for the *E.coli* termination protein Tus (Hill *et al.*, 1989); and (ii) arrest of the leading strand synthesis in an *in vitro* replication system containing the purified *E.coli* termination and primase proteins occurs mainly within T2 (Hill and Marians, 1990).

Lagging strand DNA synthesis is initiated within a 30 bp region of the terminus, at one main and two minor sites (Figures 5 and 6). The heterogeneity of initiation sites could be due to a low specificity of the primosome (Ogawa *et al.*, 1983) and possibly also to the variable length of RNA primers which might still be attached to DNA. Approximately 50–100 bases of the lagging strand template

are not paired at the terminus (Figure 6), which is consistent with the observations reported for the *B.subtilis* chromosome (Williams and Wake, 1989).

It is likely that the large plasmids pSM19035 (27.5 kb) and pIP501 (29.7 kb), isolated from group A and B streptococci, respectively (Malke, 1974; Horodniceanu *et al.*, 1976), also replicate by a theta mechanism since the replication regions of these plasmids are highly homologous to that of pAMβ1 (Sorokin and Khazak, 1989; Brantl *et al.*, 1989; Swinfield *et al.*, 1990). Preliminary data support this hypothesis (E.Le Chatelier, personal communication). Further work is required to establish whether other large plasmids from Gram positive bacteria use a similar mode of replication. The approaches developed here and elsewhere (Bruand *et al.*, 1990) could be helpful in this respect.

We have previously shown that pAMβ1 is structurally stable (Jannière *et al.*, 1990). The two related large plasmids, pSM19035 and pIP501, also appear to be structurally stable, since large DNA segments have been successfully cloned and maintained in vectors derived thereof (Rabinovich *et al.*, 1985; Scherwitz-Harmon and McKay, 1987). In contrast, small plasmids from Gram positive bacteria are most often structurally unstable (Gruss and Ehrlich, 1989). These observations point to a correlation between plasmid size and structural stability. It is tempting to speculate that the mode of replication underlies this correlation, large and stable plasmids using the theta mechanism whereas small and unstable plasmids use the rolling circle mechanism. The relationship between mode of replication and structural stability might also hold for Gram negative bacteria, since in *E.coli* theta replicating plasmids like ColE1 are structurally more stable than rolling circle replicating phages (Hermann *et al.*, 1980; Zinder and Boeke, 1982).

Materials and methods

Bacterial strains and plasmids

B.subtilis strains MI112 (*leuA8 arg15 thr5 recE4 hsdR hsdM*; Tanaka and Sakaguchi, 1978) and SB202 (*trpC2 tyrA1 aroB2 hisH2*; this laboratory) were used. Plasmids pWS10, pIL206 and pIL253 were described previously (Smith *et al.*, 1985; Simon and Chopin, 1988). Plasmid pIL253-ter was constructed by joining a 1.9 kb *Cla*I segment of pWS10 carrying the replication terminus of the *B.subtilis* chromosome to *Eco*RI and *Bam*HI cleaved pIL253, after rendering both blunt-ended by Klenow polymerase. Coordinates are according to Swinfield *et al.* (1990) for pAMβ1 and to Carrigan *et al.* (1987) for the *B.subtilis* terminus.

Microbiological and enzymatic techniques

Bacterial cells were grown in Luria broth, supplemented when necessary with 0.3 μg/ml of erythromycin. Induction of competence and transformation were described previously (cf. Jannière *et al.*, 1990). Restriction enzymes were used as recommended by suppliers. To relax the accumulated pIL253-ter replication intermediates purified in CsCl gradients, DNase I was used at a ratio of 100 pg to 20 ng of DNA in a 20 μl reaction mixture, which was 100 mM in Tris−HCl pH 8, 5 mM in $MgCl_2$ and 0.5 mM in EDTA. After 10 min of incubation at 30°C the reaction was arrested by addition of EDTA to 10 mM. A kit supplied by Boehringer-Mannheim was used for dephosphorylation and ^{32}P-labelling of 5′ ends. Sequenase kit from USB was used for sequencing reactions.

DNA preparation and analysis

To detect accumulation of replication intermediates of pIL253-ter and to analyse replication intermediates of pIL206 by agarose gel electrophoresis, total DNA of the strains which harboured the plasmid of interest was prepared as described by te Riele *et al.* (1986a). To purify pIL253-ter replication intermediates, the total DNA was prepared as follows. Exponentially growing cells (OD_{650} = 0.7) were harvested by low-speed centrifugation from a 500 ml culture and resuspended in 20 ml of a buffer containing 25% sucrose, 0.1 M NaCl, 1 mM EDTA and 50 mM Tris−HCl, pH 8.4 ml of a solution containing 10 mg/ml of lysozyme were added. After 20 min of incubation at 37°C, 1.2 ml of 0.5 M EDTA were added. The suspension was kept on ice for 15 min, 25 ml of 2% SDS were added and the incubation on ice was continued for 15 min. 13 ml of 5 M NaCl were then added and the lysate was left at 4°C overnight. It was then centrifuged at 18 000 r.p.m. for 30 min in a Beckman JA-20 rotor at 4°C. The supernatant was collected and the DNA was de-proteinized, precipitated and purified on CsCl−ethidium bromide gradients with standard methods (Maniatis *et al.*, 1982). Replication intermediates of pIL253-ter accumulate between the supercoiled and relaxed DNA bands as a consequence of their intermediate superhelicity. To extrude the newly synthesized strands by branch migration, the *Pst*I cleaved replication intermediates were incubated at 80°C for 1 h and cooled slowly to room temperature.

Two-dimensional gels were run according to Brewer and Fangman (1987). Transfer of DNA from the gels onto nitrocellulose filters, hybridization and preparation of radioactively labelled probes were performed as described by Maniatis *et al.* (1982).

Samples for electron microscopy were prepared as described by Westmoreland *et al.*, (1969). The size of molecules was measured with a Biocom 200 image analyser.

Acknowledgements

We thank M.Smith for generously providing plasmid pWS10, D.Halpern for excellent technical help, J.Hejna and M.A.Petit for assistance with the electron microscopy technique, G.Wake, P.Noirot, B.Michel, J.Hejna, S.Gruss and C.Anagnostopoulos for helpful comments on the manuscript, and V.Akveson and F.Haimet for artwork assistance. C.B. was supported by a Ministère de la Recherche et de la Technologie fellowship. This investigation was supported, in part, by grants from Commission des Communautés Européennes (BAP-0141-F) and Fondation pour la Recherche Médicale.

References

Baas,P. and Jansz,H. (1988) *Curr. Topics Microbiol. Immunol.*, **136**, 31−70.

Bramhill,D. and Kornberg,A. (1988) *Cell*, **54**, 915−918.

Brantl,S., Nowak,A., Behnke,D. and Alonso,J.C. (1989) *Nucleic Acids Res.*, **17**, 10110.

Brefort,G., Magot,M., Ionesco,H. and Sebald,M. (1977) *Plasmid*, **1**, 52−66.

Brewer,B.J. and Fangman,W.L. (1987) *Cell*, **51**, 463−471.

Brewer,B.J. and Fangman,W.L. (1988) *Cell*, **55**, 637−643.

Brewer,B.J., Sena,E.P. and Fangman,W.L. (1988) *Cancer Cells*, **6**, 229−234.

Bruand,C., Ehrlich,S.D. and Jannière,L. (1990) In Zukowski,M.M., Ganesan,A.T. and Hoch,J.A. (eds), *Genetics and Biotechnology of Bacilli*. Academic Press, New York, Vol. 3, pp. 123−129.

Carrigan,C.M., Haarsma,J.A., Smith,M.T. and Wake,R.G. (1987) *Nucleic Acids Res.*, **15**, 8501- 8509.

Clewell,D.B., Yagi,Y., Dunny,G.M. and Schultz,S.K. (1974) *J. Bacteriol.*, **117**, 283−289.

Dasgupta,S., Masukata,H. and Tomizawa,J.-I. (1987) *Cell*, **51**, 1113−1122.

Ehrlich,S.D., Noirot,P., Petit,M.-A., Jannière,L., Michel,B. and te Riele,H. (1986) In Setlow,J.K. and Hollaender,A. (eds), *Genetic Engineering*. Plenum Publishing Corporation, New York, Vol. 8, pp. 71−83.

Garnier,T. and Cole,S.T. (1988) *Plasmid*, **19**, 151−160.

Gros,M.-F., te Riele,H. and Ehrlich,S.D. (1987) *EMBO J.*, **6**, 3863−3869.

Gros,M.-F., te Riele,H. and Ehrlich,S.D. (1989) *EMBO J.*, **8**, 2711−2716.

Gruss,A. and Ehrlich,S.D. (1989) *Microbiol. Rev.*, **53**, 231−241.

Herrmann,R., Neugebauer,K., Pirkl,E., Zentgraf,H. and Schaller,H. (1980) *Mol. Gen. Genet.* **177**, 231−242.

Hill,T.M. and Marians,K.J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2481−2485.

Hill,T.M., Pelletier,A.J., Tecklenburg,M.L. and Kuempel,P.L. (1988) *Cell*, **55**, 459−466.

Hill,T.M., Tecklenburg,M.L., Pelletier,A.J. and Kuempel,P.L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1593−1597.

Horaud,T., Le Bouguenec,C. and Pepper,K. (1985) *J. Antimicrob. Chemother.*, **16**, 111- 135.

Horiuchi,T. and Hidaka,M. (1988) *Cell*, **54**, 515−523.

Horiuchi,T., Hidaka,M., Akiyama,M., Nishitani,H. and Sekiguchi,M. (1987) *Mol. Gen. Genet.*, **210**, 394−398.

Horodniceanu,T., Bouanchaud,D.H., Bieth,G. and Chabbert,Y.A. (1976) *Antimicrob. Agents Chemother.*, **10**, 795−801.

Imanaka,T., Fujii,M. and Aiba,S. (1981) *J. Bacteriol.*, **146**, 1091–1097.
Imanaka,T., Ishikawa,H. and Aiba,S. (1986) *Mol. Gen. Genet.*, **205**, 90–96.
Jannière,L., Bruand,C. and Ehrlich,S.D. (1990) *Gene*, **87**, 53–61.
Lewis,P.J., Ralston,G.B., Christopherson,R.I. and Wake,R.G. (1990) *J. Mol. Biol.*, **214**, 73–84.
Linskens,M.H.K. and Huberman,J.A. (1988) *Mol. Cell. Biol.*, **8**, 4927–4935.
Malke,H. (1974) *Mol. Gen. Genet.*, **135**, 349–367.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miyazaki,C., Kawai,Y., Ohtsubo,H. and Ohtsubo,E. (1988) *J. Mol. Biol.*, **204**, 331–343.
Model,P. and Russel,M. (1988) In Calender,R. (eds) *The Bacteriophages* Plenum Publishing Corp., New York, pp. 375–456.
Novick,R.P. (1989) *Annu. Rev. Microbiol.*, **43**, 537–565.
Ogasawara,N., Fujita,M.Q., Moriya,S., Fukuoka,T., Hirano,M. and Yoshikawa,H. (1990) In Drlica,K. and Riley,M. (eds), *The Bacterial Chromosome*. A.S.M., Washington, pp. 287–295.
Ogawa,T., Arai,K.-I. and Okazaki,T. (1983) *J. Biol. Chem.*, **258**, 13353–13358.
Pelletier,A.J., Hill,T.M. and Kuempel,P.L. (1989) *J. Bacteriol.*, **171**, 1739–1741.
Rabinovich,P.M., Haykinson,M.Y., Arutyunova,L.S., Yomantas,Y.V. and Stepanov,A.I. (1985) In Helinski,D.R., Cohen,S.N., Clewell,D.B., Jackson,D.A., Hollaender,A. (eds) *Plasmids in Bacteria*. Plenum Press, New York and London, pp. 635–656.
Scherwitz-Harmon,K. and McKay,L.L. (1987) *Appl. Environ. Microbiol.*, **53**, 1171–1174.
Simon,D. and Chopin,A. (1988) *Biochimie*, **70**, 559–566.
Smith,M.T. and Wake,R.G. (1988) *J. Bacteriol.*, **170**, 4083–4090.
Smith,M.T., Aynsley,C. and Wake,R.G. (1985) *Gene*, **38**, 9–17.
Sorokin,A.V. and Khazak,V.E. (1989) In Butler,L.O., Harwood,C. and Moseley,B.E.B. (eds), *Genetic Transformation and Expression*. Intercept Ltd, Andover, UK, pp. 269–281.
Sozhamannan,S., Dabert,P., Moretto,V., Ehrlich,S.D. and Gruss,A. (1990) *J. Bacteriol.*, **172**, 4543- 4548.
Swinfield,T.-J., Oultram,J.D., Thompson,D.E., Brehm,J.K. and Minton,N.P. (1990) *Gene*, **87**, 79–90.
Tanaka,T. and Sakaguchi,K. (1978) *Mol. Gen. Genet.*, **165**, 269–276.
te Riele,H., Michel,B. and Ehrlich,S.D. (1986a) *Proc. Natl. Acad. Sci. USA*, **83**, 2541–2545.
te Riele,H., Michel,B. and Ehrlich,S.D. (1986b) *EMBO J.*, **5**, 631–637.
Weiss,A.S. and Wake,R.G. (1984) *Cell*, **39**, 683–689.
Westmoreland,B.C., Szybalski,W. and Ris,H. (1969) *Science*, **163**, 1343–1348.
Williams,N.K. and Wake,R.G. (1989) *Nucleic Acids Res.*, **17**, 9947–9956.
Zinder,N.D. and Boeke,J.D. (1982) *Gene*, **19**, 1–10.

Received on March 5, 1991; revised on April 23, 1991