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A DNA sequence outside the pUB110 minimal replicon is required for normal replication in *Bacillus subtilis*

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ABSTRACT

The origin of lagging strand synthesis in pUB110, oriL, has been localized within 140 bases outside the pUB110 minimal replicon. The oriL DNA sequence is a cis-acting and orientation dependent determinant required for normal plasmid replication. Rearrangements affecting oriL cause plasmid instability, lead to the accumulation of replication intermediates and result in a marked reduction of the plasmid copy number in some recombination deficient mutant strains. In addition, deletion of oriL triggers a dnaB-dependent mode of replication. Insertion of the functionally asymmetric oriL region in the proper orientation into pC194 reduces the accumulation of single-stranded DNA during the replication of this plasmid.

INTRODUCTION

pUB110 is a 4.5 Kb multicopy plasmid carrying neomycin (kanamycin) and phleomycin (bleomycin) resistance determinants. The plasmid has been widely used in recombinant DNA experiments in *Bacillus subtilis*.

Previously, we had identified a 1.2 kb pUB110 segment required for driving autonomous replication in *B. subtilis* Rec^+ cells. This region can be divided into three functionally discrete segments: i) a 24 bp region that acts as the initiation origin (oriU), ii) the 949 bp determinant of the essential replication initiation protein, repU, and iii) a 358 bp incom- patibility region, incA, overlapping with the repU gene (1). This, however, is not the basic replicon in the definition of Kollek et al. (2). While in Rec^+ cells plasmids containing the minimal replication region are present at wild type copy number (basic replicon ?), their copy number is drastically reduced in recE4 cells (3, this communication).

We report here on the identification of a region outside the

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previously defined minimal replicon (1) that works as a lagging strand origin (oriL) and is required for normal replication. Analysis of this region suggests that pUB110 replicates via a rolling-circle mechanism with at least two alternative mechanisms for priming lagging strand synthesis.

MATERIALS AND METHODS

Strains and plasmids.

Bacillus subtilis strains YB1015, *rec*E4; BG125, *add*A5; BG81, *stb*-2; BG193, *dna*B37(Ts); BG197, *dna*D23(Ts) and BG204, *dna*B19(Ts) *rec*E4 belong to an isogenic set of DNA recombination or replication mutants constructed in the YB886 genetic background (4, this communication). The genotype of YB886 is *trp*C2 *met*B5 *amy*E *xin*-1 *att*SPβ. Strains YB965, *pol*A5 and BG191 *pol*A5 *add*A5 are isogenic and their genotype is *trp*C2 *met*B5 *his*B *xin*-1 *att*SPβ (3, 4). The *dna*B37(Ts) (5) and *dna*D23(Ts) (6) mutations were placed into the YB886 "prophage-free" genetic background by plasmid mediated selection as described previously (4).

The naturally occuring plasmids pC194 (7) and pUB110 (8) have been used. pUB110 derivatives generated by *in vitro* deletion of the small *Eco*RI-*Pvu*II fragment (pBT30), the small *Pvu*II-*Nci*I fragment (pBT31), the small *Pvu*II-*Tha*I fragment (pBT32), and the small *Eco*RI-*Nci*I fragment (pBT33) have been previously described (1, Fig. 1). By *in vitro* deletion of the small *Tha*I-*Nci*I fragment of pUB110 we generated plasmid pBT49. The *Pvu*II-*Hae*III pUB110 fragment containing the *ori*L region was joined to the filled in *Hin*dIII site of pC194 generating plasmids pBT46 and pBT47 in orientations I and II, respectively.

Transformation, transduction and DNA techniques.

The method of Rottländer and Trautner (9) was used for transforming *B.subtilis* competent cells.

Plasmid DNAs were prepared on preparative and analytical scales as described before by Alonso and Trautner (10). Crude DNA lysates were prepared from exponentially growing cells essentially as described by Viret and Alonso (3). For the analysis of the *dna*B37(Ts) strain the overnight culture was diluted 1/100 into fresh medium and grown at 30°C to about 2.5×10^7 cells per

ml. At this point, the culture was quickly transferred (in less than 2 min) to 46°C and was allowed to grow for an additional 120 minutes. Aliquots of cultures were harvested and crude DNA lysates were prepared.

Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer, Mannheim (FRG) or from BRL, Gaithersburg, Md. (U.S.A.) and were used as specified by the manufacturer.

Plasmid stability tests.

Plasmid segregational stability was tested as described previously (4).

Southern hybridization, determination of the relative plasmid copy numbers and replication rates.

For Southern analysis, the crude DNA lysates were separated by gel electrophoresis in 0.7% agarose. When necessary, two gels loaded with identical samples were run in parallel and transferred to GeneScreen nylon membranes (NEN Research products) either with or without prior depurination and denaturation essentially as described previously (3). In non-denaturing conditions, only single-stranded DNA can be retained on the membrane. Twice CsCl-ethidium bromide purified and nick- translated pUB110 DNA was used as a probe.

Relative plasmid copy number was determined as described previously (1). The replication rates given in the text refer to the intensities of the plasmid bands present on the auto- radiograms with respect to time zero. The linearity of the response with respect to DNA concentration was checked using control bands of DNA at five different concentrations.

Quantitative scans performed on a laser densitometer (LKB Ultro Scan XL) were integrated by using the LKB GelScan XL software package. Corrections were performed for the fact that double-stranded molecules give twice as intense hybridization signals as single-stranded molecules of the same size.

Computer analysis.

RNA secondary structures were predicted by using the computer software package of the University of Wisconsin Genetic Computer Group in a Vax computer (11).

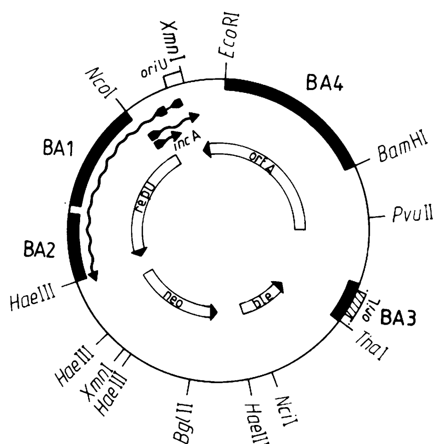

Fig.1.
Physical structure of pUB110. The inner closed bars represent the membrane binding sites (BA), the outer open bar the oriU and the outer dashed bar the oriL region. The wavy lines represent putative RNA transcripts and IncA indicates the incompatibility region. Internal arrows indicate the open reading frames (McKenzie et al.1986, Bashkirov et al. 1896, Davis et al. 1987, Maciag et al. submitted). The relevant restriction sites are depicted.

RESULTS.

Identification and mapping of oriL, the pUB110 lagging strand replication region (conversion signal) in B. subtilis repair-deficient cells.

Maciag et al. (1) have mapped the determinants for the negative, IncA, and the positive, RepU, trans-acting products of pUB110 and defined the minimal region that is necessary and sufficient for autonomous plasmid replication (coordinates 3118-4316 of McKenzie et al.,12) in Rec$^+$ cells. The RepU-dependent replication origin, termed oriU, has been localized within a 24 bp segment at coordinates 4293-4316 that lies upstream of the RepU coding region (1, Fig. 1).

pUB110 presents some particular physiological features not present in other plasmids in Bacillus subtilis : i) during replication in rec$^+$ cells, it shows very little accumulation of single stranded circular DNA, SS(c) DNA (13), ii) it is segregationally stable in all Rec$^-$ mutant strains tested (4), and iii)

four DNA regions (BA1 - BA4) in pUB110 have a membrane binding affinity (10, see Fig. 1).

To test if regions at or near the DNA membrane binding sites affect plasmid copy number as well as the accumulation of replication intermediates, in vitro deletions of those sites were generated and an analysis of the different plasmid forms was carried out.

As reported previously, pUB110 in Rec^+ cells shows very low levels of SS(c) DNA (see 13). We assume that SS(c) material is circular as reported by te Riele et al. (13) for plasmid pC194. Its sensitivity to endonuclease S1 has been confirmed (data not shown).

The pUB110 copy number determined for exponentially growing B. subtilis Rec^+ cells is about 46 to 50 copies of the double-stranded form and 2 to 3 copies of the single-stranded form per cell (see Fig. 2 lane 1). pUB110 derivatives such as pBT30, lacking the BA4 region with the subsequent truncation of the orfA (see Fig. 1), are characterized by lower levels of SS(c) DNA independent of the genetic background (Fig.2 lanes 2 and 6). In addition, we observed that both pUB110 and pBT30 (data not shown) SS(c) DNA is nearly absent (1.7- fold reduction) in the stb-2 mutant strain (Fig. 2 lane 14).

Deletion of the BA3 region (pBT31, pBT32 and pBT33) or its inversion (3) leads to the accumulation of SS(c) DNA in all the Rec^- mutants tested as well as in the wild type strain YB886. Thus, we are tempted to assume that the origin of lagging strand synthesis (oriL) is located within the 516 bp DNA segment deleted in plasmid pBT32. In addition, as previously reported (3), a novel plasmid form corresponding to linear double or single stranded multigenomic plasmid molecules (hmw) is observed in wild type and addA5 genetic backgrounds when BA3 is deleted. Since the hmw forms are apparently generated by a break-dependent type of replication independent of the plasmid RepU effector (Fig. 2, see ref. 3), we have tested the hmw accumulation in different DNA repair mutant strains. From those studies we observed that the synthesis of hmw plasmid DNA differs from normal plasmid replication in that it requires at least DNA polymerase I and RecE, as well as the Stb product(s) (see Fig. 2).

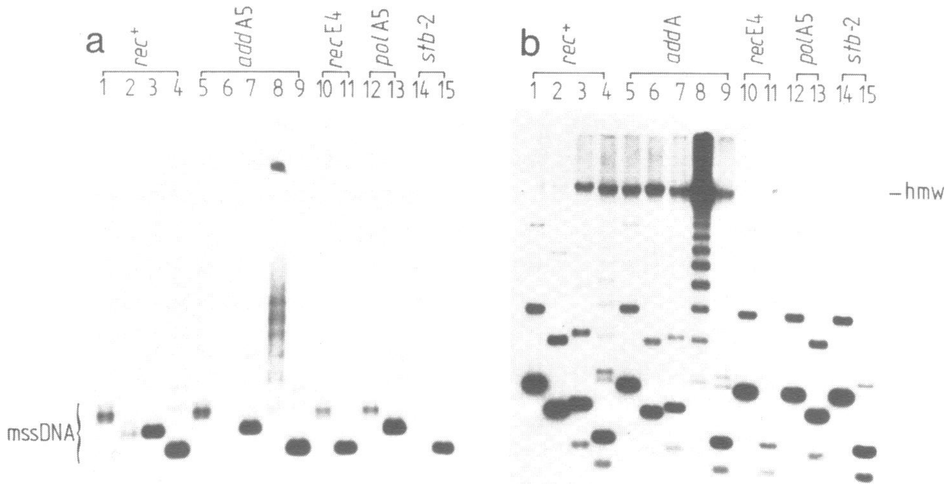

Fig. 2.
Plasmid forms of pUB110 and derivatives in different genetic backgrounds. The DNA was not-denatured (a) or denatured (b) prior transfer to the nylon membrane and hybridized to ^{32}P-labeled pUB110 DNA. mssDNA and hmw refer to the position of single-stranded unit length covalently closed and multigenome-length linear molecules, respectively. Lanes 1, 5, 10, 12 and 14: plasmid pUB110; lanes 2 and 6: pBT30; lanes 3, 7 and 13: pBT31 and lanes: 4, 9, 11, and 15: plasmid pBT33. Lane 8: pBT82 with pBT33 as a helper. Volumes of lysates were adjusted to equal amounts of chromosomal DNA.

Figure 2 shows that neither pUB110 plasmid copy number nor the amount of SS(c) DNA is affected in YB1015 (a recE4 strain). However, plasmids lacking the BA3 region have a copy number reduction of about 4- to 5- fold in YB1015 whereas the ratio of SS(c) DNA to the covalently closed circular form (CCC) is 3- to 4- fold higher than in Rec$^+$ cells (see Fig 2 lanes 4 and 11). Is this effect attributable to poor viability of the recE4 strain? Since one-third of the pC194 plasmid copies were SS(c) DNA (13, our unpublished results) and the copy number is about the same in both Rec$^+$ and recE4 genetic backgrounds (data not shown) we have ruled out a toxic effect that may lead to poor viability.

Recently we have reported that plasmid pUB110 regulates its copy number by an antisense RNA that controls the synthesis of the rate limiting RepU protein (1, our unpublished results).

However, despite the reduced copy number of pBT31, the accumulation of SS(c) DNA observed in the recE4 strain indicates that the number of initiation events for plasmid replication seems to be constant (see Fig. 2 lanes 4 and 11).

Introduction of plasmid pBT33 into the ATP-dependent DNase (addA5) mutant strain gave rise to two types of colonies: very slowly growing ones that carried pBT33 in a form phenotypically indistinguishable from the one observed in the Rec^+ genetic background and possibly resulting from mutations suppressing the AddAB complex deficiency (Fig. 2 lane 9), and more healthly growing colonies bearing non-autonomously replicating plasmids in addition to pBT33. One of those, pBT82, arose by an in vivo deletion between two homology boxes consisting of 6 base pairs (1). One of the deletion endpoints is located within the pUB110 oriU replication origin. The deletion regenerates a functional replication origin, at least for initiation of plasmid replication. pBT82 does not resolve to monomers and shows a ladder of both double and single-stranded DNA forms (Fig. 2, lane 8). By electron microscopic analysis, we have found that the majority of the oligomeric forms of pBT82 shown in Figure 2b lane 8 are true multimers without catenated or knotted structures (data not shown). The single-stranded DNA ladder (Fig. 2a lane 8) could result from a defect at the level of termination of strand displacement due to the loss, in the deletion event, of a fully functional termination recognition site. This observation further supports the assymmetric mode of pUB110 replication which could use two overlapping but distinct regions, one for initiation and the other for termination of leading strand synthesis as reported for at least one filamentous coliphage (see ref. 15).

From these results we infer: i) pUB110 replicates asymmetrically like other Gram positive plasmids, such as pT181 (16), pC194 (3, 17) and pE194 (3), ii) the conversion signal for lagging strand synthesis (oriL) is located within the 516 bp BA3 segment and about 2.7 kb from the RepU target origin (oriU), iii) the oriL region does not contain an autonomous replication origin, iv) when oriL is inactivated by deletion or inversion an alternative mechanism is necessary to convert the SS(c) DNA to the double-stranded form and this seems to require the host re-

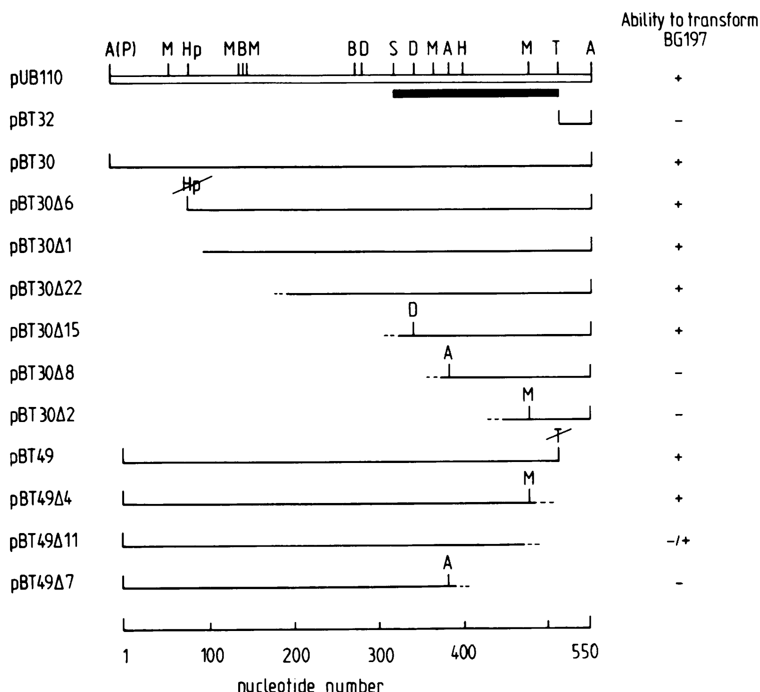

Fig. 3.
Deletion mapping of oriL. The open bar shows the restriction map of the 550 bp AluI fragments E and F where oriL has been localized (see text). The filled bar denote the membrane-binding site 3 (BA3) and the thin line represents plasmids containing deletions. Abbreviations: A, AluI; B,BspMI; D,DraI; H,HinfI; Hp, HpaII; M,MnlI; S,Sau3AI and T,ThaI. (P) indicates the PvuII overlapping with the AluI.

combination functions, v) deletion of the four binding sites (pBT82) does not block plasmid replication, and vi) plasmid pBT82 might have lost by *in vivo* deletion part of the nucleotide sequence required for efficient termination of leading strand DNA synthesis.

Deletion Mapping of oriL.

During the construction of a set of *B. subtilis* isogenic *dna* (Ts) mutant strains in a prophage free genetic background (Alonso et al. submitted), we have observed that oriL$^-$ plasmids do not transform some mutant strains, or do so at a very low frequency, at the permissive temperature. Those strains were impaired in genes coding for different subunits of the DNA polymerase III

core enzyme, namely dnaD23 and dnaF133 (see 18, Yoshikawa pers. comm.).

By in vitro deletion analysis we have located oriL within the 516 bp PvuII-ThaI segment. The inability of plasmids lacking this region to transform the dnaD23 mutant strain made it possible to dissect the oriL region further.

pUB110 was linearized either with PvuII or ThaI and eroded with Bal31 exonuclease for various times. The generated products were then digested with EcoRI or NciI, respectively, and their ends filled in with Klenow polymerase. The resulting large fragments were ligated and used to transform wild type B. subtilis competent cells. The in vitro deletion of the small EcoRI-PvuII or ThaI-NciI fragments generated the control plasmids pBT30 and pBT49, respectively.

The resulting transformant plasmids that were smaller than the ones not treated with Bal31 (pBT30 and pBT49) were designated pBT30Δ or pBT49Δ and selected for further analysis. Figure 3 shows the restriction map of the pBT30Δ and pBT49Δ plasmids.

Plasmids pUB110, pBT30, pBT30Δ6, pBT30Δ1 pBT30Δ22 and pBT30Δ15 or pBT49 and pBT49Δ4 transform the dnaD23 (BG197) mutant strain with identical frequency as the wild type, whereas plasmids pBT30Δ2, pBT30Δ8 and pBT49Δ7 fail to transform BG197. Removal of about 20 to 30 bp at the ThaI restriction site or about 340 bp at the PvuII site does not affect the transformation potential of those plasmids. This localizes oriL within a fragment of about 140 bp between coordinates 1380 to 1520. Since some of the deleted plasmids show an intermediate transformation frequency (see pBT49Δ11), we believe that this region may be still smaller.

Is pUB110 lagging strand origin effective also in the pC194 context ?

In spite of the high degree of homology between pC194 and pUB110 either at the oriU DNA sequence or the Rep protein levels (see ref. 1), they show a distinct pattern with respect to the amount of SS(c) DNA that is accumulated during replication (see 13). In order to test if this difference could be attributable to the efficiency of usage for each lagging strand origin, we cloned pUB110 oriL into pC194. Both plasmids show the same directionality of replication; indeed the displaced strand corresponds to

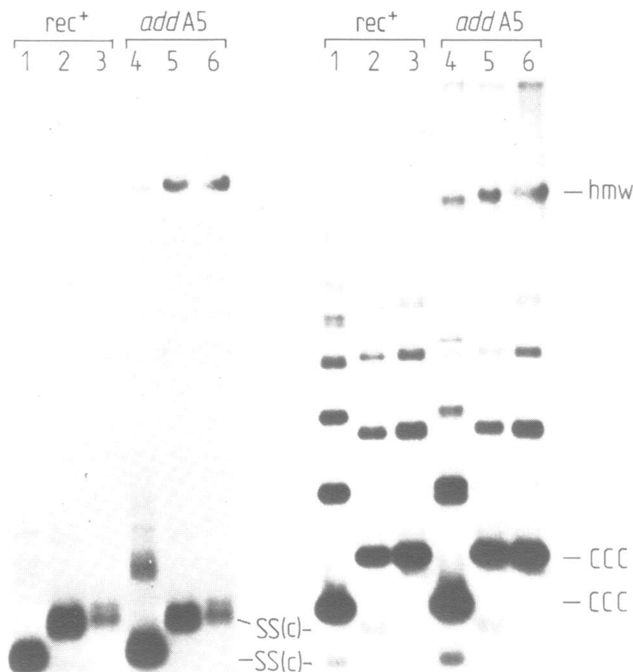

Fig. 4.
Plasmid forms in preparations of pC194, pBT46 and pBT47 in the YB886 rec+ and BG125 addA5 genetic backgrounds. The loading and transfer conditions are as described in legend of fig. 2 and the hybridization was to pC194 nick translated DNA. Lanes 1 and 4: pC194; lanes 2 and 5: pBT46 and lanes 3 and 6: plasmid pBT47. The high molecular weight, hmw, covalently closed circular double-stranded, CCC, and single-stranded circular SS(c), plasmid forms are indicated.

the non-coding one of the Rep effector (see 19, 13). Assuming that pUB110 oriL is preferentially utilized over the native lagging strand origin of pC194 (termed pal, 20) we expected to be able to modify the pattern of SS(c) DNA accumulated by the latter provided oriL was functional in this context and its orientation was suitable for priming.

To test this hypothesis, the pUB110 PvuII-HaeIII oriL region between coordinates 1034 and 2081 was cloned, in both orientations, in the filled-in HindIII site of pC194, generating plasmids pBT46 and pBT47. By analogy with the organization of pUB110, only pBT47 has the proper orientation for priming.

Figure 4 shows the hybridization patterns of identical samples transferred to nylon membranes without (Fig. 4a) or with denaturation prior to transfer (Fig. 4b). As previously reported (3), the signals in Figure 4a correspond to SS(c) DNA in Rec^+ cells or SS(c) and single stranded hmw DNA in the addA5 strain. Plasmid pC194 and pBT46 (oriL in the inverse orientation) accumulate large amounts of SS(c) DNA without any appreciable copy number defect, whereas plasmid pBT47 shows 4- to 5- fold less SS(c) DNA and a proportional increase in CCC DNA.

In Figure 4b a signal corresponding to SS(c) DNA, with a faster electrophoretic mobility than the supercoiled double-stranded DNA is observed for plasmid pC194 and pBT46 as well as double-stranded hmw DNA which accumulated in the addA5 genetic background as previously reported (3).

te Riele et al. (13) have suggested that SS(c) DNA is involved in preventing plasmid oligomerization, which has been shown to cause the loss of plasmids. However, it has also been reported that the segregational instability of pC194 in the addA5 mutant strain strongly corresponded to the reduced growth rate of plasmid-harboring cells, when compared to that of plasmid-free segregants, possibly as a result of a "physiological stress"(4). On the other hand, pUB110 is stably inherited in this strain (4). To test if the accumulation of SS(c) DNA might be associated with the physiological stress of pC194-bearing cells we used the pC194 derivatives pBT46 and pBT47 (see above).

The segregation stability of plasmids pBT46 and pBT47 was analysed during approximately 100 generations of growth without selective pressure in the addA5 background. As reported for pC194, about 18% of the viable cells are pBT46-free at the beginning of non-selective growth, and segregation (about 1% per generation) is observed from the first generation on without a detectable lag period. In contrast, plasmid pBT47 is 55- fold more stable than pBT46. Furthermore, plasmid pBT47 transforms the addA5 mutant strain with a 4- fold higher frequency than pBT46 when selection for phleomycin is applied.

We conclude from these data: i) that oriL is functionally asymmetric in both pUB110 and pC194, ii) that the oriL conversion signal is independent of the plasmid initiator protein, iii) that

oriL does not require a finite distance relative to the Rep-dependent origin and, iv) the accumulation of SS(c) DNA form is asociated with plasmid instability (see 4).

The host dnaB function is required for replication of pUB110 oriL$^-$ plasmid derivatives.

In Bacillus subtilis, the best studied DNA replication initiation mutations have been those mapping in the dnaB locus. Those mutants have been divided in two classes dnaBI [dnaB1(Ts), dnaB37 (Ts)] and dnaBII [dnaB19(Ts)]. No dnaB equivalent has been so far identified in E. coli (see ref. 21).

Upon a temperature shift up of dnaB mutants the initiation of chromosomal DNA replication stops concurrently with the selective release of the replication origin from the cell membrane in both dnaBI and dnaBII mutants whereas pUB110 replication is affected only in the dnaBI mutant strain (22, 23).

Recently, it has been reported that both double- and single-stranded plasmid forms are released from the membrane at the non-permissive temperature in the dnaBI class mutants whereas only double stranded DNA is released in the dnaBII class mutants (24).

If the dnaB function is actually required for initiation of pUB110 replication, a replication block is expected at high temperature. If on the other hand this function, whether in a membrane associated or in a cytoplasmic manner, is required in the conversion step from SS(c) to double-stranded DNA, after temperature shift up the amount of SS(c) DNA (replication intermediates) should increase. Accordingly, a dnaBI class mutant strain, dnaB37(Ts), carrying either pC194, pUB110 or pBT32 (oriL$^-$) was analyzed at permissive (30°C) and nonpermissive (46°C) temperatures for the amount of the different plasmid forms.

As shown in Fig. 5, the amount of pUB110 double-stranded CCC DNA increased 3.6- fold during 120 min of incubation at 46°C, as compared to 3- fold in the case of pC194 (data not shown), whereas the concentration of SS(c) DNA is nearly constant with respect to that of the double stranded form (5% and 27% respectively). Our results differ from those of Winston and Sueoka (22), perhaps due to the fact that they have used a pUB110 derivative in which priming at the oriL region might be affected.

At the permissive temperature, pBT32 shows a wild type copy

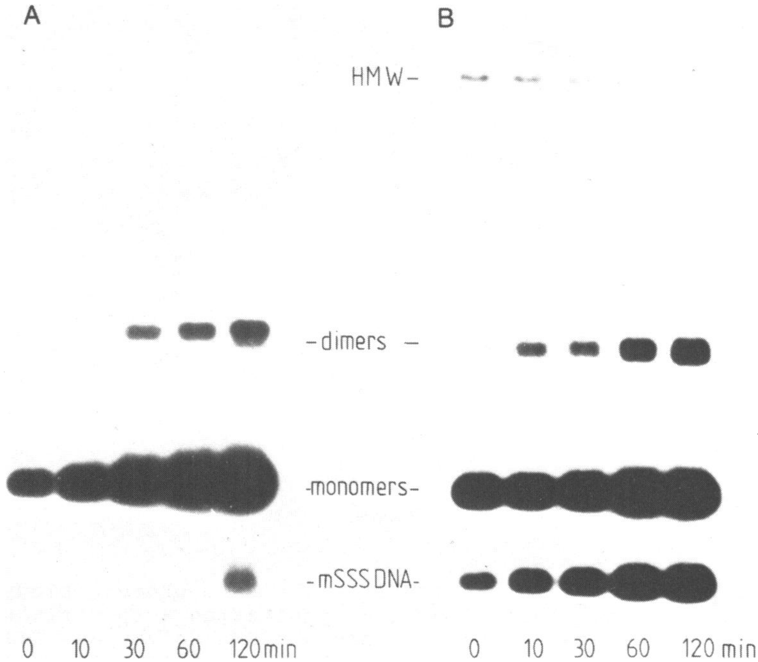

Fig. 5.
Effect of dnaB37 on the replication of plasmids pUB110 (A) and pBT32 (B). Samples were withdrawn at the time of the shift up (0) and after 10, 30, 60 and 120 min at 46° C and processed as described in Fig. 2 for denaturated DNA.

number but a 5- fold increased amount of SS(c) DNA relative to pUB110. After 120 min. at the nonpermissive temperature however, the double stranded form increased only 1.2- fold while SS(c) DNA increased 2.4- fold.

From this experiment we infer that: i) neither pUB110 nor pC194 strictly require the DnaB function for replication, ii) the dnaB gene product is not required for the initiation of plasmid replication, iii) thermal inactivation of dnaB in oriL⁻ plasmids leads to the accumulation of SS(c) DNA. Therefore the activity of a conversion signal other than oriL depends on the DnaB protein(s).

DISCUSSION.

We have previously localized the pUB110 origin of replication, oriU, within a 24 bp segment at nucleotides 4293 to 4316. The

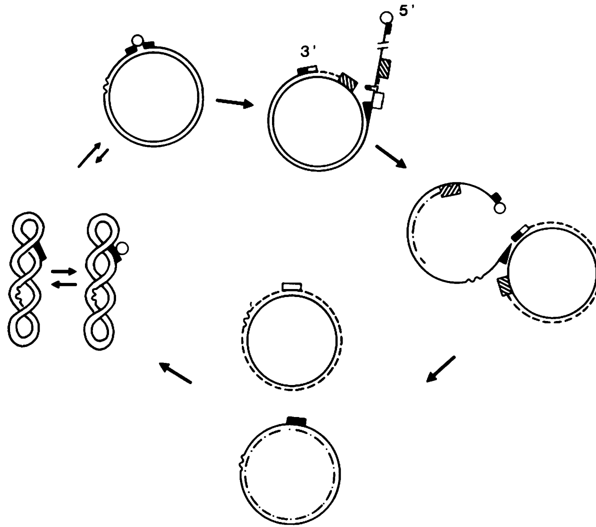

Fig. 6.
Model of pUB110 plasmid replication. The RepU protein binds to the pUB110 superhelical DNA origin, generating a specific nick and a "functional gap". DNA helicase and DNA polymerase III account for asymmetric replication. RepU terminates DNA synthesis after a full round of replication. Broken lines represent DNA polymeraseIII-dependent newly synthesized DNA initiated at the RepU-dependent 3′ -end, dotted broken lines correspond to the DNA polymeraseIII-dependent chain elongation at primed 3′-end Primase-dependent lagging strand origin. O Plasmid-encoded RepU protein, DNA polymeraseIII, DNA helicase, Primosome, SSB, and leading strand origin and lagging strand origin.

plasmid-encoded protein effector, RepU, was shown to bind to the oriU DNA region, to trigger initiation of plasmid replication (1). In this report, we have defined the major lagging strand origin, oriL, within a 140 nucleotide segment. Furthermore, we have shown that the oriL region does not constitute on its own an origin of replication *in vivo*, since pUB110 derivatives fused to a thermosensitive replicon are unable to form colonies at non-permissive temperature when either the repU open reading frame was truncated or oriU was deleted *in vitro* (1).

We envision the following mechanism of plasmid DNA replication (Fig. 6): i) the rate limiting RepU protein binds to supercoiled plasmid DNA at the 24 bp leading strand origin region, oriU (1, our unpublished results), ii) it promotes a single strand cleav-

age and iii) it remains attached to the 5′ terminus (protected end) during the strand displacement (see 3). iv) the RepU protein may provide a "functional gap" which allows a 3′ to 5′ DNA helicase to bind and initiate unwinding of DNA, whereas DNA Polymerase III may account for asymmetric plasmid replication. Since in the cell single-stranded DNA is always complexed with a DNA-binding protein (as the E. coli SSB) and such a complex may be formed as soon as the single-stranded DNA is generated, SSB is likely to be required for pUB110 replication.

v) After one full round of replication, RepU terminates the strand displacement by cleaving the regenerated oriU to produce unit length SS(c) DNA (3, our unpublished results) similar to the ΦX174 bacteriophage (25). Interestingly, the oriL$^-$ mini-plasmid pBT82 lacking all four membrane binding sites shows a ladder of single- and double-stranded multimeric forms of DNA. From this result we can infer that initiation at oriU does not require the BA sites and that oriU may be composed of two overlapping but distinct domains, one for initiation and the other for termination of leading strand synthesis (affected in pBT82) as reported for one filamentous coliphage (see ref. 15).

vi) Initiation of the complementary strand synthesis, which takes place 2.7 kb from the RepU target site, seems to occur via an uncharacterized priming mechanism, as soon as the specific site (oriL region) is exposed (1). Indeed, pUB110 exhibits very little accumulation of SS(c) (about 3% to 5% of the total plasmid DNA) provided the oriL site is present in the proper orientation. This property is independent of the Rep-dependent plasmid origin and the distance between both origins (see fig 3, plasmid pBT47). In the case of pC194, which naturally accumulates high amounts of single stranded DNA (13), insertion of the oriL region in the proper orientation eliminates the accumulation of this material and segregationally stabilizes the plasmid. The simplest explanation for this effect is that oriL of pUB110 is utilized more efficiently than the pal lagging strand origin of pC194 (20, 26)and that the SS(c) accumulation leads to plasmid instability.

Within the 140 bp oriL region, a hairpin structure with a calculated free energy of formation of - 70 KJ per mol is predicted,

which might be functionally relevant. No DNA consensus sequences for the E. coli n′ or DnaA box have been found.

In addition to the oriL-dependent mechanism, our results support the existence of an alternative mechanism of priming lagging strand synthesis for pUB110 , perhaps through a DNA-membrane association complex involving the dnaB product. Indeed, the $oriL^-$ plasmid pBT32 replicating either in the dnaB37(Ts) or dnaB19(Ts) (data not shown) mutant strains accumulates SS(c) DNA at the nonpermissive temperature and stops lagging strand DNA synthesis, suggesting that the alternative priming mechanism is DnaB-dependent. In the E. coli system, it has recently been reported that the host DnaA protein binds to the pBR322 origin region via a DnaA box, by-passing the requirement for primosome assembly sites by directing DnaB, DnaC and DnaG proteins to the origin (27).

On the other hand, in Rec^+ cells $oriL^-$ plasmids are still able to undergo conversion from SS(c) to double-stranded DNA without reduction of plasmid copy number. In this situation, conversion to double-stranded DNA could occur through a recombinational intermediate, similar to that observed for bacteriophage T_4 (28), since this step requires the RecE product (see Fig. 2 lane 11). This could be part of a third conversion mechanism to generate a replication fork which requires the RecE protein. Interestingly, a synergistic effect on plasmid replication intermediates was observed on the dnaB19 recE4 genetic background.

Plasmid pUB110 is stably maintained in B. subtilis recombination-deficient strains (4), whereas cells harboring pUB110 derivatives lacking oriL, or with oriL in its inverted orientation, are lost through competition with plasmid-free cells, mainly as a result of the shorter generation time of the latter (3). In the present report, we show that the unstable pC194 replicon can be stabilized through insertion of oriL, in addA5 cells, by reducing the accumulation of SS(c) DNA form (plasmid pBT47).

Little is known about the molecular mechanism involved in plasmid stabilization. However, we can envisage that the segregational instability of seg^- pC194 derivatives (10, 4, 3, our unpublished results) or pUB110 derivatives lacking oriL may be due to either the generation of deleterious replication intermediates

(hmw, SS(c)) or by the depletion of a given host function(s) or the association of both (3, Kupsch et al. to be published elsewhere).

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