The replicative polymerases PolC and DnaE are required for theta replication of the *Bacillus subtilis* plasmid pBS72

Marina Titok,¹ Catherine Suski,² Bérengère Dalmais,³ S. Dusko Ehrlich³ and Laurent Jannière^{3†}

Correspondence

Laurent Jannière

laurent.janniere@jouy.inra.fr

¹Belarussian State University, Biological Faculty, Department of Genetics and Biotechnology, Minsk 220050, 4 Scorina Avenue, Belarus

²Memorial Sloan-Kettering Cancer Center, NY 10021, USA

³Laboratoire de Génétique Microbienne, Bâtiment des Biotechnologies, INRA, 78352 Jouy en Josas, France

Plasmids are the tools of choice for studying bacterial functions involved in DNA maintenance. Here a genetic study on the replication of a novel, low-copy-number, *Bacillus subtilis* plasmid, pBS72, is reported. The results show that two plasmid elements, the initiator protein RepA and an iteron-containing origin, and at least nine host-encoded replication proteins, the primosomal proteins DnaB, DnaC, DnaD, DnaG and DnaI, the DNA polymerases DnaE and PolC, and the polymerase cofactors DnaN and DnaX, are required for pBS72 replication. On the contrary, the cellular initiators DnaA and PriA, the helicase PcrA and DNA polymerase I are dispensable. From this, it is inferred that pBS72 replication is of the theta type and is initiated by an original mechanism. Indirect evidence suggests that during this process the DnaC helicase might be delivered to the plasmid origin by the weakly active DnaD pathway stimulated by a predicted interaction between DnaC and a domain of RepA homologous to the major DnaC-binding domain of the cellular initiator DnaA. The plasmid pBS72 replication fork appears to require the same functions as the bacterial chromosome and the unrelated plasmid pAM β 1. Most importantly, this replication machinery contains the two type C polymerases, PolC and DnaE. As the mechanism of initiation of the three genomes is substantially different, this suggests that both type C polymerases might be required in any Cairns replication in *B. subtilis* and presumably in other bacteria encoding PolC and DnaE.

Received 17 November 2005

Revised 20 December 2005

Accepted 17 January 2006

INTRODUCTION

DNA replication is a ubiquitous biological process carried out by proteins that have evolved profoundly since the last common ancestor. For key elements like initiators, helicases, primases, catalytic DNA polymerases and processivity factors, their evolution has been so extensive that they are postulated to originate from peptides that diverged beyond recognition or from different proteins (Edgell & Doolittle, 1997; Giraldo, 2003; Leipe *et al.*, 1999). Surprisingly, this diversity, obvious when comparing Eubacteria to Archaea and Eukarya, is also manifest among bacterial species. For instance, in the well characterized eubacteria *Escherichia coli* and *Bacillus subtilis*, the primosomes required for initiating replication at origins or collapsed replication forks have been shown to greatly differ in their constitution and mechanism of helicase loading (Bruand *et al.*, 1995; Cox

et al., 2000; Davey & O'Donnell, 2003; Lemon *et al.*, 2002; Marsin *et al.*, 2001; Polard *et al.*, 2002; Velten *et al.*, 2003). Differences have also been documented in the DNA polymerase III (Pol III) holoenzyme, a subassembly of the replication machinery where DNA synthesis is catalysed. In *E. coli* this element contains 10 different subunits of which four (θ , γ , χ and ψ) are missing in *B. subtilis* (Bruck & O'Donnell, 2000; Bruck *et al.*, 2002; Lemon *et al.*, 2002; Martinez-Jimenez *et al.*, 2002; McHenry, 2003).

Based on systems for which a Pol III holoenzyme has been isolated and/or reconstructed *in vitro*, it is generally thought that both DNA strands of genomes are polymerized by a single replicative polymerase of the C (prokaryotes) or B (eukaryotes) families, dimerized in a holoenzyme structure (for reviews, see Benkovic *et al.*, 2001; Bullock, 1997). However, this scheme might not be universal as replication in numerous organisms depends on two different polymerases. For instance, in *B. subtilis* and related bacteria of the *Bacillus/Clostridium* and *Thermotogales* groups, two type C

[†]On the CNRS staff.

Abbreviation: CN, copy number.

polymerases (PolC and DnaE) are essential for chromosome replication and colocalize with the replication machinery (Dervyn *et al.*, 2001; Inoue *et al.*, 2001; Lemon & Grossman, 1998, 2000). In the replication fork of the *B. subtilis* plasmid pAM β 1, PolC might be mainly involved in leading strand synthesis while DnaE might be required for efficient lagging strand polymerization (Dervyn *et al.*, 2001). This polymerase specialization detected in a plasmid system might also apply to chromosomal forks as (i) initiation of pAM β 1 replication depends on the cellular machinery that initiates DNA synthesis at collapsed replication forks (Bruand *et al.*, 1995, 2001; Marsin *et al.*, 2001; Polard *et al.*, 2002) and (ii) a bioinformatic study showed that bacteria containing both PolC and DnaE have a much stronger gene strand bias than DnaE⁺ and PolC⁻ species (Rocha, 2002). This latter point suggests that asymmetrical replication forks could have a major role in organizing bacterial chromosomes. DNA replication also depends on two different replicative polymerases (Pol δ and Pol ϵ , B family) in numerous eukaryotic cells (for reviews, see Burgers, 1998; Hubscher *et al.*, 2000; Johnson & O'Donnell, 2005; Kawasaki & Sugino, 2001; Sugino, 1995). As in bacterial forks, these polymerases might be specialized as several data suggest that Pol ϵ would elongate leading strand synthesis while Pol δ would synthesize the lagging strand (see, for instance, Fukui *et al.*, 2004; Garg *et al.*, 2004; Karthikeyan *et al.*, 2000; Morrison & Sugino, 1994; Shcherbakova & Pavlov, 1996). Recently, it was proposed that chromosomal replication in archaea might also depend on two distinct DNA polymerases (Henneke *et al.*, 2005).

To further test the requirement for PolC and DnaE in Gram-positive bacteria, we undertook a genetic study of the replication of *B. subtilis* plasmid pBS72 (Titok *et al.*, 2003). Results showed that pBS72 replication (i) is of the theta-type, (ii) depends on two plasmids elements, an initiator protein and an origin of replication, (iii) initiates by an original mechanism independent of the cellular initiators DnaA and PriA and (iv) is carried out by a replication fork indistinguishable from that of the *B. subtilis* chromosome, including PolC and DnaE. These data illustrate again how plasmids capture for their own benefit cellular factors and support our previous proposal that the catalytic centre of the *B. subtilis* replication machinery might include two different type C polymerases.

METHODS

Bacterial strains and growth conditions. *B. subtilis* strains used were: 168 (*trpC2*) (Costa Anagnostopoulos); L1430 (*ilvA metC lys-21*), L1432 (*dnaB19 metC ilvA*), L1433 (*dnaC14 metC lys-21*), L1434 (*dnaD23 metC lys-21*), L1435 (*dnaE20 metC ilvA*), L1437 (*dnaG5 metC lys-21*) and L1439 (*dnaI2 metC ilvA*) (Mauel & Karamata, 1984); 1A74 (*dnaX8132 hisA1 thr-5*), 1A224 (*hisH2 pheA1 trpC2*) and 1A226 (*polA5 hisH2 pheA1 trpC2*) (Bacillus Genetic Stock Center); EDJ51 (168 *dnaE2.10 ins-phl*), EDJ58 (168 *dnaE2.6 ins-phl*), HVS609 (168 *spac-1::polC emr* containing pMAP65) and HVS614 (168 *spac-1::dnaE emr* containing pMAP65) (Dervyn *et al.*, 2001); PB1669 (168 *dnaF69*) (Attolini *et al.*, 1976); CRK6000 (*purA16*

metB5 hisA3 guaB) and NIS6200 (CRK6000 *proA::pBPA21::cat*) (Hassan *et al.*, 1997); NIS6311 [CRK6000 *spoIII::pBPA21::cat dnaA(ochre)*] (Shigeki Moriya, University of Technology, Sydney, Australia); HVS606 (168 *pcrA3 ins* of pMAP55 in pBR322-Cm^R inserted at the *metD* locus) (Petit *et al.*, 1998); PPBJ120 (168 *priA1::pAPJ11::erm*) and PPBJ65 (168 *priA::pAPJ12::erm*, PriA⁺) (Polard *et al.*, 2002). Hosts for plasmid constructions were regular *E. coli* strains. Growth in a rich medium (LB), induction of competence and transformation were as described previously for *B. subtilis* (Bron, 1990) and for *E. coli* (Sambrook *et al.*, 1989). To construct the Δ *priA* strain, 168 competent cells harbouring pMTLBS72 (Titok *et al.*, 2003) were transformed to erythromycin resistance with total DNA prepared from PPBJ120. Transformants were isolated and propagated in a poor medium (Polard *et al.*, 2002). Antibiotics were used at the following concentrations (μ g ml⁻¹): ampicillin, 100; chloramphenicol, 6; erythromycin, 0.6; phleomycin, 2. To investigate the effect of PolC inhibition by the drug HB-EMAU (Wright *et al.*, 2005) fresh cells harbouring pMTLBS72 were grown in the presence of various concentrations (0, 0.625, 1.25, 2.5 and 5 μ M) of the drug for about 1.5 h. In these conditions, cell growth was slightly reduced at 1.25 μ M and arrested at ≥ 2.5 μ M HB-EMAU (not shown). Filamentation was observed at ≥ 1.25 μ M.

DNA manipulation and plasmid construction. DNA preparation and manipulation were carried out essentially according to standard procedures (Bron, 1990; Sambrook *et al.*, 1989). Previously described plasmids used were pMTL20C, pMTL21E (Swinfield *et al.*, 1991) and pMTLBS72 (a pMTL20C derivative containing a *Bgl*II fragment carrying the pBS72 replicon; Fig. 1, top line) (Titok *et al.*, 2003). Derivatives of pMTLBS72 carrying a truncated or mutated pBS72 replicon (Fig. 1) were generated in *E. coli* by incubating the parental plasmid with restriction enzymes cleaving in the cloned fragment and, when appropriate, in the flanking polylinker (segments were blunt-ended with the Klenow enzyme when required). The plasmids yielded were termed pLJH16-18 and pLJH67-76. The DnaA requirement was investigated with the pBS72 derivative pLJH13 composed of the pBS72 *Bgl*II fragment of pMTLBS72 cloned at the *Bgl*II site of the erythromycin-resistant *E. coli* vector pMTL21E.

Copy number (CN) measurement. To determine CN variation in replication mutants, total DNA was extracted, restricted, run in an agarose gel and analysed by Southern analysis using as probes radiolabelled plasmid and chromosomal sequences. Molecular Dynamic tools were used to quantify CN variations as described previously (Titok *et al.*, 2003). CN values presented in Table 1 were calculated from three independent measurements. Control experiments showed that the integrated plasmid replicon (pLS32) that directs chromosomal replication in the Δ *dnaA* strain (Hassan *et al.*, 1997) does not affect pBS72 derivative CN values (not shown). CN measurement in the Δ *priA* strain was carried out in LB with fresh transformants isolated in a poor medium. Since the strain is genetically unstable (Bruand *et al.*, 2001; Polard *et al.*, 2002), the PriA⁻ phenotype was confirmed genetically at the same time as DNA preparation (not shown).

RESULTS

Plasmid replication functions

We previously cloned and sequenced a 3.1 kb replication determinant widely represented in large (≥ 90 kb) plasmids of a Belarus collection of *B. subtilis* strains (Titok *et al.*, 2003). The segment, fused to a pUC-like plasmid to form pMTLBS72, contains three complete ORFs (Fig. 1, top). The product of ORF-1 (hereafter termed RepA) might correspond to the plasmid initiator as it contains a putative

Fig. 1. Genetic localization of the pBS72 replication determinants. (a) Mutation analysis. The 3081 bp replicon (*Bgl*II fragment) contains three complete ORFs and one truncated ORF (horizontal arrows in black and grey, respectively), conventional expression signals (promoters, bent arrows; transcription terminators, lollipops) and a putative origin of replication (*ori*). ORF1 (*repA*), ORF-2, ORF-3 and ORF-4 (C-terminal part of a truncated protein) encode proteins of 344, 168, 113 and 87 aa, respectively. Relevant restriction sites are indicated. Deleted or mutated (*) pBS72 replicons were isolated in *E. coli* and tested for transformation activity in *B. subtilis*. Constructs displaying a high transformation activity and CN similar to that of the original plasmid pMTLBS72 are indicated by '+'. The construct having a low transformation efficiency and a reduced CN is pinpointed by '±'. Derivatives that gave no transformants are indicated by '-'. The shortest segment required for optimal replication (*Cla*I-*Eco*RI) is represented by a thicker line and is carried by plasmid pLJH76. (b) Salient features of the origin region. Arrows R1-R5 indicate putative RepA-binding sites. Black and grey bars indicate AT- and GC-rich sequences, respectively. The DnaA-binding site is boxed. Brackets delimit the sequence deleted in pLJH74.

DNA-binding motif (HTH motif between amino acids 111 and 132) and a C-terminal region (position 276-330) homologous (35 % identity, 63 % similarity) to the central part of the N-terminal domain I of the highly conserved chromosomal initiator DnaA. In *E. coli*, this internal region of domain I (position 13-68) is mainly involved in stable binding to the replicative helicase (Felczak *et al.*, 2005; Seitz *et al.*, 2000; Sutton *et al.*, 1998). The ~400 bp downstream sequence would include the plasmid origin as it contains typical hallmarks of such replication elements: repeated sequences (iterons), DnaA-binding sites and an AT-rich region (for reviews, see del Solar *et al.*, 1998; Espinosa *et al.*, 2000; Helinski *et al.*, 1996). The function of the remaining two ORFs, which have no homologues in databases, is not known.

To genetically identify plasmid sequences required for replication, pMTLBS72 derivatives carrying parts of the 3 kb replicon were constructed and assessed for replication activity in *B. subtilis* by a transformation assay. Results (Fig. 1) showed that the minimal segment capable of

autonomous replication is 1929 bp in length (pLJH76). It encompasses *repA* and the downstream ~400 bp region. As expected, the *repA* product is essential for replication as a 5' deletion of the coding frame and a truncation of the protein by frameshift mutations at the *Nsi*I or *Sal*I sites abolish transformation activity (pLJH16-18 and pLJH67-68). The ~400 bp downstream sequence is also essential for replication since (i) its deletion (pLJH71 and pLJH75) causes a complete loss of plasmid replication and (ii) its alteration by a small (32 bp) deletion changing the spacing between two repeats (pLJH74) sharply reduces the transformation activity and the CN value of the replicon. These results suggest that RepA is the plasmid initiator and that the ~400 bp includes the plasmid origin. Note that none of the functional derivatives (except pLJH74) displayed a marked change in CN (not shown). This indicates that ORF-2 and ORF-3 are not needed for replication control and that the CN of the replicon might be regulated at the level of *repA* expression and/or by origin handcuffing (del Solar *et al.*, 1998; Espinosa *et al.*, 2000; Helinski *et al.*, 1996; Park *et al.*, 2001).

Host-encoded replication functions

To search for host-encoded proteins required for plasmid replication, pMTLBS72 was introduced into wild-type and isogenic mutants affected in DNA polymerases PolC (*dnaF69*), DnaE (*dnaETs2.6* and *2.10*) or Pol I (*polA5*), the processivity factor DnaN (*dnaG5*), the replication machinery organizer DnaX (*dnaX8132*), the initiator proteins DnaA (Δ *dnaA*) and PriA (Δ *priA*), the replicative helicase DnaC (*dnaC14*), the DnaC loaders DnaB, DnaD and DnaI (*dnaB19*, *dnaD23* and *dnaI2*), the primase DnaG (*dnaE20*) and the PcrA helicase required for rolling-circle replication and involved in Cairns replication (*pcrA3*) (for reviews, see Khan, 2005; Lemon *et al.*, 2002; Moriya *et al.*, 1999; Winston, 1982; Yoshikawa & Wake, 1993). Transformants were readily obtained at 30 °C with the complete set of strains. To measure the effect of T mutations on plasmid replication, total DNA was extracted before and after a short growth (about five generations) at the semi-restrictive temperature. The DNA was then restricted, run in agarose gels and analysed by Southern blotting and hybridization, using radiolabelled plasmid DNA and a chromosomal PCR

fragment as probes. The ratios of plasmid/chromosomal signals were then determined before and after growth at the semi-permissive temperature and compared. In the case of null or constitutive mutations (Δ *dnaA*, Δ *priA*, *polA5* and *pcrA3*), plasmid/chromosomal ratios were measured at 37 °C and compared to those of the isogenic wild-type strains grown at the same temperature. Replica-plating analysis showed that the plasmid was maintained in the cell population throughout the experiment in most of the mutants. However, for the *dnaB19* and, to a lesser extent, *dnaI2* mutants, cured cells were detected at the end of growth in proportions reaching 0.5 and 0.3, respectively. The proportion of plasmid-free cells was taken into account for CN determination.

In most mutants, a moderate (two- to threefold) CN reduction was observed at semi-restrictive temperature (Fig. 2 and Table 1). Plasmid replication is thus more affected than chromosome replication in these strains, showing that the corresponding mutated factors are required for pBS72 replication. This applies to the polymerases PolC and DnaE, the processivity factor DnaN, the replication machinery

Fig. 2. Plasmid CN measurement in various replication mutants. Total DNA of wild-type (WT) and mutated strains grown in permissive or semi-permissive conditions was analysed by Southern analysis after *Pst*I restriction (except in the case of *dnaG5* and *dnaX8132* where *Ssp*I was used) and hybridization with radiolabelled probes homologous to the plasmid (P) and a chromosomal (C) segments (see text for more details). Plasmid pMTLBS72 was used in all the strains except Δ *dnaA* and the wild-type isogenic cell where a related plasmid (pLJH13) was used. Results obtained within the 168^(a) and HVS325^(b) wild-type strains are shown as references for 168- and HVS325-derived strains.

Table 1. Plasmid CN measurement in various replication mutants: quantitative analysis

Tested protein	Mutation	Relative CN*
None	None	1 ± 0.18
Replication fork proteins		
PolC	<i>dnaF69</i>	0.57 ± 0.05
DnaE	<i>dnaE2.6</i>	0.33 ± 0.12
DnaE	<i>dnaE2.10</i>	0.27 ± 0.02
DnaX	<i>dnaX8132</i>	0.27 ± 0.05
DnaN	<i>dnaG5</i>	0.60 ± 0.08
Primosomal proteins		
DnaB	<i>dnaB19</i>	0.29 ± 0.13
DnaC	<i>dnaC14</i>	0.31 ± 0.08
DnaD	<i>dnaD23</i>	0.30 ± 0.1
DnaG	<i>dnaE20</i>	0.25 ± 0.05
DnaI	<i>dnaI2</i>	0.33 ± 0.17
DnaA	Null	2.41 ± 0.64
PriA	Null	6.43 ± 2.89
Other proteins		
Pol I	<i>polA5</i>	1.11 ± 0.15
PcrA	<i>pcrA3</i>	1.08 ± 0.28

*Relative mean CN values from three independent experiments ± SEM are shown. Values obtained in the strain 168 are given. In the HVS325 WT strain, the relative CN was 0.9 ± 0.09. The CN in the WT context is 5.9 ± 1.1 (Titok *et al.*, 2003).

organizer DnaX and the primosomal proteins DnaB, DnaC, DnaD, DnaG and DnaI. In strains lacking DnaA or PriA (because of gene deletion) or encoding a replication-defective form of the polymerase Pol I (*polA5* mutation; Laipis & Ganesan, 1972) or an inactive form of the PcrA helicase (*pcrA3*; Anand *et al.*, 2005), the CN is either unchanged (Pol I and PcrA mutants) or increased (DnaA and PriA mutants). These factors are hence not required for pBS72 replication. As the DnaA and PriA null strains filament (Hassan *et al.*, 1997; Polard *et al.*, 2002; our observations), the CN increase observed in these contexts might result from dilution of negative factors in enlarged cells allowing plasmid amplification. However, in the DnaA context, it cannot be ruled out that the CN increase results from alleviation of a negative effect of DnaA on plasmid replication, because DnaA might bind to *dnaA* boxes of the plasmid origin or to host-encoded factors limiting for plasmid replication. Finally, it should be noted that (partial) inactivation of required replication factors did not always cause CN reduction. In several instances, the (partial) inactivation kept the CN unchanged. This occurs, for instance, when PolC inactivation is caused by the *dnaF33* mutation or when cells are grown in the presence of the PolC-inhibitory compound HB-EMAU (not shown). A similar result was obtained in cells depleted of the wild-type PolC or DnaE enzymes because of the weak expression of the corresponding gene expressed from the IPTG-inducible *spac-I* promoter (not shown). This probably results from a

simultaneous arrest of plasmid and chromosome replication. Finally, the lack of single-stranded production in any PolC and DnaE replication mutant precluded the determination of whether these enzymes are specialized in strand synthesis (not shown).

DISCUSSION

The genetic analysis presented here has uncovered major aspects of the replication of *B. subtilis* plasmid pBS72. First, two sets of data show that it replicates as Cairns molecules rather than rolling circles. Like Cairns-type genomes, pBS72 replication depends on the major replicative helicase DnaC, helicase loaders DnaB, DnaD and DnaI, and its origin carries typical signatures of theta-replicating genomes [iterons, an AT-rich sequence and *dnaA* boxes; Titok *et al.*, 2003; this work (for reviews, see del Solar *et al.*, 1998; Espinosa *et al.*, 2000; Helinski *et al.*, 1996)]. On the contrary, unlike rolling-circle-replicating plasmids, (i) pBS72 is independent of PcrA helicase activity, (ii) it lacks any homology with rolling-circle plasmids which are classified into a dozen groups based on sequence similarity, (iii) none of its derivatives accumulate single-stranded DNA as do generally derivatives of rolling-circle genomes and (iv) pBS72 is much larger (~90 kb) than any rolling-circle plasmids (≤10 kb) [Titok *et al.*, 2003; this work (for reviews, see del Solar *et al.*, 1998; Espinosa *et al.*, 2000; Khan, 2005)].

The second aspect of replication uncovered by this study concerns the mechanism of initiation of pBS72 replication. The goal of this replication step is to load the replicative helicase on the single-stranded DNA generated close to the initiator/DNA complex. In bacteria, two main initiation pathways have been described so far. The PriA-dependent pathway is the best characterized in *B. subtilis*. It is required to restart DNA synthesis at disassembled replication forks. Dissection of the pathway by genetic and biochemical means revealed the following scheme. First, PriA binds DNA forks carrying single-stranded DNA coated with single-stranded DNA-binding protein and recruits DnaD. The complex then assists the binding of DnaB which recruits DnaI and DnaC. This cascade of reactions yields helicase DnaC loading on the appropriate single-stranded DNA by a ring-assembly mechanism (Bruand *et al.*, 2001, 2005; Marsin *et al.*, 2001; Polard *et al.*, 2002; Velten *et al.*, 2003). The second initiation pathway, the DnaA-dependent pathway, operates at the chromosomal origin of replication. It requires a DnaA/ori interaction as an initial step followed by a cascade of reactions involving DnaB, DnaC, DnaD and DnaI (for reviews, see Lemon *et al.*, 2002; Yoshikawa & Wake, 1993). It is speculated that this cascade is similar to that occurring in the PriA-dependent pathway (Bruand *et al.*, 2005; Velten *et al.*, 2003). It is interesting to note here that DnaD interacts with DnaA and PriA (Bruand *et al.*, 2005; Ishigo-Oka *et al.*, 2001; Marsin *et al.*, 2001). This interaction might thus play a key role in bringing DnaB, DnaI and DnaC to the initiator/DNA complexes.

The factors required for pBS72 initiation of replication are two plasmid elements, the origin and the RepA protein, and four host-encoded replication factors, namely DnaD, DnaB, DnaI and DnaC. It is, however, surprisingly independent of the initiator proteins DnaA and PriA (so far, only *oriβ* from R6K, R1, pLS32, pAMβ1 and RSF1010 have been shown to be DnaA-independent; Bernander *et al.*, 1991; Espinosa *et al.*, 2000; Hassan *et al.*, 1997; Kelley & Bastia, 1992; Scherzinger *et al.*, 1991; Tang *et al.*, 1989). This suggests that the recruitment of the DnaD-driven cascade to the RepA/origin complex might differ from that occurring at the DnaA/*oriC* and PriA/DNA complexes. A simple model would be that the single-stranded DNA generated by RepA at the plasmid origin is used as a landing pad for DnaD which has a single-stranded DNA-binding activity (Bruand *et al.*, 2005; Marsin *et al.*, 2001). In support of this hypothesis, it was previously shown that the DnaC helicase can be loaded on long stretches of single-stranded DNA via a weakly efficient DnaA- and PriA-independent pathway that requires DnaD, DnaB and DnaI (the DnaD pathway) (Bruand *et al.*, 2005; Marsin *et al.*, 2001). However, similar to all initiator/DNA complexes studied in detail so far [for reviews, see del Solar *et al.*, 1998; Helinski *et al.*, 1996], RepA must not generate a single-stranded region large enough to activate the DnaD pathway at the origin. Additional factors should come into play to compensate for this. Interestingly, several studies show that plasmid initiators interact with host-encoded replication proteins for initiating DNA synthesis [RepA of pSC101 (Datta *et al.*, 1999; Sharma *et al.*, 2001), TrfA of RK2 (Pacek *et al.*, 2001) and π of R6K (Abhyankar *et al.*, 2004; Lu *et al.*, 1998; Ratnakar *et al.*, 1996); for a review, see Konieczny, 2003]. Thus, it is conceivable that RepA interacts with replication factors to stimulate the DnaD pathway. One possible interacting protein would be DnaD itself as this enzyme is already known to interact with the initiators DnaA and PriA (Bruand *et al.*, 2005; Ishigo-Oka *et al.*, 2001; Marsin *et al.*, 2001). However, RepA carries a region homologous to domain I of DnaA-type proteins which, in the *E. coli* enzyme, strongly binds the replicative helicase and is required for stabilizing it in the prepriming complex at *oriC* (Felczak *et al.*, 2005; Seitz *et al.*, 2000; Sutton *et al.*, 1998). Hence, instead of, or in addition to, interacting with DnaD, RepA may bind to DnaC, acting in place of DnaA to efficiently recruit and stabilize the helicase in the plasmid prepriming complex, increasing the efficiency of the DnaD pathway.

The final aspect of pBS72 replication uncovered by this analysis is that once the prepriming complex has formed, the DnaG primase is recruited for synthesizing RNA primers, opening a route for the assembly of the replication machinery. Actually, this machinery is indistinguishable from that acting on the chromosome and the plasmid pAMβ1 [Bruand *et al.*, 1995; Ceglowski *et al.*, 1993; Dervyn *et al.*, 2001 (for reviews, see Lemon *et al.*, 2002; Winston, 1982; Yoshikawa & Wake, 1993)]. It includes the two DNA polymerases PolC and DnaE, the replication machinery organizer DnaX and the processivity factor DnaN. According to studies carried

out in *Streptococcus pyogenes* (Bruck & O'Donnell, 2000), it should also contain the δ and δ' proteins. The lack of mutations in these genes has precluded their study in this work. Hence, three different genomes (pBS72, pAMβ1 and the *B. subtilis* chromosome) are replicated by related, and possibly identical, machinery requiring two different type C polymerases, PolC and DnaE. The substantial differences in the mechanism of DnaC loading in the three genomes (the DnaA pathway at the chromosomal origin, the PriA pathway in pAMβ1 and putatively the DnaD pathway in pBS72) suggest that this machinery operates in any Cairns replication in *B. subtilis* and presumably also in related bacteria of the *Bacillus/Clostridium* and *Thermotogales* groups.

ACKNOWLEDGEMENTS

We thank Shigeki Moriya for providing Δ *dnaA* and isogenic strains, Alexandro Galizzi for strain PB1669, Patrice Polard for the Δ *priA* strain, and N. C. Brown and G. E. Wright for making available HB-EMAU. Nathalie Galleron is thanked for her excellent technical assistance in DNA sequencing and Emmanuelle Le Chatelier, Danielle Cancelli and David Lane for stimulating discussions and critical reading of the manuscript. The work was supported, in part, by a research fund from INTAS (INTAS97-1464).

REFERENCES

- Abhyankar, M. M., Reddy, J. M., Sharma, R., Bullesbach, E. & Bastia, D. (2004). Biochemical investigations of control of replication initiation of plasmid R6K. *J Biol Chem* **279**, 6711–6719.
- Anand, S. P., Chattopadhyay, A. & Khan, S. A. (2005). The PcrA3 mutant binds DNA and interacts with the RepC initiator protein of plasmid pT181 but is defective in its DNA helicase and unwinding activities. *Plasmid* **54**, 104–113.
- Attolini, C., Mazza, G., Fortunato, A., Ciarrocchi, G., Mastromei, G., Riva, S. & Falaschi, A. (1976). On the identity of *dnaP* and *dnaF* genes of *Bacillus subtilis*. *Mol Gen Genet* **148**, 9–17.
- Benkovic, S. J., Valentine, A. M. & Salinas, F. (2001). Replisome-mediated DNA replication. *Annu Rev Biochem* **70**, 181–208.
- Bernander, R., Dasgupta, S. & Nordstrom, K. (1991). The *E. coli* cell cycle and the plasmid R1 replication cycle in the absence of the DnaA protein. *Cell* **64**, 1145–1153.
- Bron, S. (1990). Plasmids. In *Molecular Biological Methods for Bacillus*, pp. 75–174. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.
- Bruand, C., Ehrlich, S. D. & Janniere, L. (1995). Primosome assembly site in *Bacillus subtilis*. *EMBO J* **14**, 2642–2650.
- Bruand, C., Farache, M., McGovern, S., Ehrlich, S. D. & Polard, P. (2001). DnaB, DnaD and DnaI proteins are components of the *Bacillus subtilis* replication restart primosome. *Mol Microbiol* **42**, 245–255.
- Bruand, C., Velten, M., McGovern, S., Marsin, S., Serena, C., Ehrlich, S. D. & Polard, P. (2005). Functional interplay between the *Bacillus subtilis* DnaD and DnaB proteins essential for initiation and re-initiation of DNA replication. *Mol Microbiol* **55**, 1138–1150.
- Bruck, I. & O'Donnell, M. (2000). The DNA replication machine of a gram-positive organism. *J Biol Chem* **275**, 28971–28983.
- Bruck, I., Yuzhakov, A., Yurieva, O., Jeruzalmi, D., Skangalis, M., Kuriyan, J. & O'Donnell, M. (2002). Analysis of a multicomponent thermostable DNA polymerase III replicase from an extreme thermophile. *J Biol Chem* **277**, 17334–17348.

- Bullock, P. A. (1997). The initiation of simian virus 40 DNA replication in vitro. *Crit Rev Biochem Mol Biol* **32**, 503–568.
- Burgers, P. M. (1998). Eukaryotic DNA polymerases in DNA replication and DNA repair. *Chromosoma* **107**, 218–227.
- Ceglowski, P., Lurz, R. & Alonso, J. C. (1993). Functional analysis of pSM19035-derived replicons in *Bacillus subtilis*. *FEMS Microbiol Lett* **109**, 145–150.
- Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J. & Marians, K. J. (2000). The importance of repairing stalled replication forks. *Nature* **404**, 37–41.
- Datta, H. J., Khatri, G. S. & Bastia, D. (1999). Mechanism of recruitment of DnaB helicase to the replication origin of the plasmid pSC101. *Proc Natl Acad Sci U S A* **96**, 73–78.
- Davey, M. J. & O'Donnell, M. (2003). Replicative helicase loaders: ring breakers and ring makers. *Curr Biol* **13**, R594–R596.
- del Solar, G., Giraldo, R., Ruiz-Echevarria, M. J., Espinosa, M. & Diaz-Orejas, R. (1998). Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**, 434–464.
- Dervyn, E., Suski, C., Daniel, R., Bruand, C., Chapuis, J., Errington, J., Janniere, L. & Ehrlich, S. D. (2001). Two essential DNA polymerases at the bacterial replication fork. *Science* **294**, 1716–1719.
- Edgell, D. R. & Doolittle, W. F. (1997). Archaea and the origin(s) of DNA replication proteins. *Cell* **89**, 995–998.
- Espinosa, M., Cohen, S., Couturier, M. & 7 other authors (2000). Plasmid replication and copy number control. In *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*, pp. 1–47. Edited by C. M. Thomas. London: Taylor & Francis.
- Felczak, M. M., Simmons, L. A. & Kaguni, J. M. (2005). An essential tryptophan of *Escherichia coli* DnaA protein functions in oligomerization at the *E. coli* replication origin. *J Biol Chem* **280**, 24627–24633.
- Fukui, T., Yamauchi, K., Muroya, T., Akiyama, M., Maki, H., Sugino, A. & Waga, S. (2004). Distinct roles of DNA polymerases delta and epsilon at the replication fork in *Xenopus* egg extracts. *Genes Cells* **9**, 179–191.
- Garg, P., Stith, C. M., Sabouri, N., Johansson, E. & Burgers, P. M. (2004). Idling by DNA polymerase delta maintains a ligatable nick during lagging-strand DNA replication. *Genes Dev* **18**, 2764–2773.
- Giraldo, R. (2003). Common domains in the initiators of DNA replication in Bacteria, Archaea and Eukarya: combined structural, functional and phylogenetic perspectives. *FEMS Microbiol Rev* **26**, 533–554.
- Hassan, A. K., Moriya, S., Ogura, M., Tanaka, T., Kawamura, F. & Ogasawara, N. (1997). Suppression of initiation defects of chromosome replication in *Bacillus subtilis* *dnaA* and *oriC*-deleted mutants by integration of a plasmid replicon into the chromosomes. *J Bacteriol* **179**, 2494–2502.
- Helinski, D. R., Toukdarian, A. E. & Novick, R. P. (1996). Replication control and other stable maintenance mechanisms of plasmids. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 2295–2324. Edited by F. C. Neidhart. Washington, DC: American Society for Microbiology.
- Henneke, G., Flament, D., Hubscher, U., Querellou, J. & Raffin, J. P. (2005). The hyperthermophilic euryarchaeota *Pyrococcus abyssi* likely requires the two DNA polymerases D and B for DNA replication. *J Mol Biol* **350**, 53–64.
- Hubscher, U., Nasheuer, H. P. & Syvaoja, J. E. (2000). Eukaryotic DNA polymerases, a growing family. *Trends Biochem Sci* **25**, 143–147.
- Inoue, R., Kaito, C., Tanabe, M., Kamura, K., Akimitsu, N. & Sekimizu, K. (2001). Genetic identification of two distinct DNA polymerases, DnaE and PolC, that are essential for chromosomal DNA replication in *Staphylococcus aureus*. *Mol Genet Genomics* **266**, 564–571.
- Ishigo-Oka, D., Ogasawara, N. & Moriya, S. (2001). DnaD protein of *Bacillus subtilis* interacts with DnaA, the initiator protein of replication. *J Bacteriol* **183**, 2148–2150.
- Johnson, A. & O'Donnell, M. (2005). Cellular DNA replicases: components and dynamics at the replication fork. *Annu Rev Biochem* **74**, 283–315.
- Karthikeyan, R., Vonarx, E. J., Straffon, A. F., Simon, M., Faye, G. & Kunz, B. A. (2000). Evidence from mutational specificity studies that yeast DNA polymerases delta and epsilon replicate different DNA strands at an intracellular replication fork. *J Mol Biol* **299**, 405–419.
- Kawasaki, Y. & Sugino, A. (2001). Yeast replicative DNA polymerases and their role at the replication fork. *Mol Cells* **12**, 277–285.
- Kelley, W. L. & Bastia, D. (1992). Activation in vivo of the minimal replication origin beta of plasmid R6K requires a small target sequence essential for DNA looping. *New Biol* **4**, 569–580.
- Khan, S. A. (2005). Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid* **53**, 126–136.
- Konieczny, I. (2003). Strategies for helicase recruitment and loading in bacteria. *EMBO Rep* **4**, 37–41.
- Laipis, P. J. & Ganesan, A. T. (1972). A deoxyribonucleic acid polymerase I-deficient mutant of *Bacillus subtilis*. *J Biol Chem* **247**, 5867–5871.
- Leipe, D. D., Aravind, L. & Koonin, E. V. (1999). Did DNA replication evolve twice independently? *Nucleic Acids Res* **27**, 3389–3401.
- Lemon, K. P. & Grossman, A. D. (1998). Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**, 1516–1519.
- Lemon, K. P. & Grossman, A. D. (2000). Movement of replicating DNA through a stationary replisome. *Mol Cell* **6**, 1321–1330.
- Lemon, K. P., Moriya, S., Ogasawara, N. & Grossman, A. D. (2002). Chromosome replication and segregation. In *Bacillus subtilis and its Closest Relatives*, pp. 73–86. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Lu, Y. B., Datta, H. J. & Bastia, D. (1998). Mechanistic studies of initiator–initiator interaction and replication initiation. *EMBO J* **17**, 5192–5200.
- Marsin, S., McGovern, S., Ehrlich, S. D., Bruand, C. & Polard, P. (2001). Early steps of *Bacillus subtilis* primosome assembly. *J Biol Chem* **276**, 45818–45825.
- Martinez-Jimenez, M. I., Mesa, P. & Alonso, J. C. (2002). *Bacillus subtilis* tau subunit of DNA polymerase III interacts with bacteriophage SPP1 replicative DNA helicase G40P. *Nucleic Acids Res* **30**, 5056–5064.
- Mauel, C. & Karamata, D. (1984). Prophage induction in thermo-sensitive DNA mutants of *Bacillus subtilis*. *Mol Gen Genet* **194**, 451–456.
- McHenry, C. S. (2003). Chromosomal replicases as asymmetric dimers: studies of subunit arrangement and functional consequences. *Mol Microbiol* **49**, 1157–1165.
- Moriya, S., Imai, Y., Hassan, A. K. & Ogasawara, N. (1999). Regulation of initiation of *Bacillus subtilis* chromosome replication. *Plasmid* **41**, 17–29.
- Morrison, A. & Sugino, A. (1994). The 3'→5' exonucleases of both DNA polymerases delta and epsilon participate in correcting errors of DNA replication in *Saccharomyces cerevisiae*. *Mol Gen Genet* **242**, 289–296.
- Pacek, M., Konopa, G. & Konieczny, I. (2001). DnaA box sequences as the site for helicase delivery during plasmid RK2 replication initiation in *Escherichia coli*. *J Biol Chem* **276**, 23639–23644.
- Park, K., Han, E., Paulsson, J. & Chatteraj, D. K. (2001). Origin pairing ('handcuffing') as a mode of negative control of P1 plasmid copy number. *EMBO J* **20**, 7323–7332.
- Petit, M. A., Dervyn, E., Rose, M., Entian, K. D., McGovern, S., Ehrlich, S. D. & Bruand, C. (1998). PcrA is an essential DNA helicase

of *Bacillus subtilis* fulfilling functions both in repair and rolling-circle replication. *Mol Microbiol* **29**, 261–273.

Polard, P., Marsin, S., McGovern, S., Velten, M., Wigley, D. B., Ehrlich, S. D. & Bruand, C. (2002). Restart of DNA replication in Gram-positive bacteria: functional characterisation of the *Bacillus subtilis* PriA initiator. *Nucleic Acids Res* **30**, 1593–1605.

Ratnakar, P. V., Mohanty, B. K., Lobert, M. & Bastia, D. (1996). The replication initiator protein pi of the plasmid R6K specifically interacts with the host-encoded helicase DnaB. *Proc Natl Acad Sci U S A* **93**, 5522–5526.

Rocha, E. (2002). Is there a role for replication fork asymmetry in the distribution of genes in bacterial genomes? *Trends Microbiol* **10**, 393.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Scherzinger, E., Haring, V., Lurz, R. & Otto, S. (1991). Plasmid RSF1010 DNA replication in vitro promoted by purified RSF1010 RepA, RepB and RepC proteins. *Nucleic Acids Res* **19**, 1203–1211.

Seitz, H., Weigel, C. & Messer, W. (2000). The interaction domains of the DnaA and DnaB replication proteins of *Escherichia coli*. *Mol Microbiol* **37**, 1270–1279.

Sharma, R., Kachroo, A. & Bastia, D. (2001). Mechanistic aspects of DnaA-RepA interaction as revealed by yeast forward and reverse two-hybrid analysis. *EMBO J* **20**, 4577–4587.

Shcherbakova, P. V. & Pavlov, Y. I. (1996). 3'→5' exonucleases of DNA polymerases epsilon and delta correct base analog induced DNA replication errors on opposite DNA strands in *Saccharomyces cerevisiae*. *Genetics* **142**, 717–726.

Sugino, A. (1995). Yeast DNA polymerases and their role at the replication fork. *Trends Biochem Sci* **20**, 319–323.

Sutton, M. D., Carr, K. M., Vicente, M. & Kaguni, J. M. (1998). *Escherichia coli* DnaA protein. The N-terminal domain and loading of DnaB helicase at the *E. coli* chromosomal origin. *J Biol Chem* **273**, 34255–34262.

Swinfield, T. J., Janniere, L., Ehrlich, S. D. & Minton, N. P. (1991). Characterization of a region of the *Enterococcus faecalis* plasmid pAM beta 1 which enhances the segregational stability of pAM beta 1-derived cloning vectors in *Bacillus subtilis*. *Plasmid* **26**, 209–221.

Tang, X. B., Womble, D. D. & Rownd, R. H. (1989). DnaA protein is not essential for replication of IncFII plasmid NR1. *J Bacteriol* **171**, 5290–5295.

Titok, M. A., Chapuis, J., Selezneva, Y. V., Lagodich, A. V., Prokulevich, V. A., Ehrlich, S. D. & Janniere, L. (2003). *Bacillus subtilis* soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector. *Plasmid* **49**, 53–62.

Velten, M., McGovern, S., Marsin, S., Ehrlich, S. D., Noirot, P. & Polard, P. (2003). A two-protein strategy for the functional loading of a cellular replicative DNA helicase. *Mol Cell* **11**, 1009–1020.

Winston, S. A. S. N. (1982). DNA replication in *Bacillus subtilis*. In *The Molecular Biology of the Bacilli*, pp. 35–69. Edited by D. Dubnau. San Diego: Academic Press Inc.

Wright, G. E., Brown, N. C., Xu, W. C., Long, Z. Y., Zhi, C., Gambino, J. J., Barnes, M. H. & Butler, M. M. (2005). Active site directed inhibitors of replication-specific bacterial DNA polymerases. *Bioorg Med Chem Lett* **15**, 729–732.

Yoshikawa, H. & Wake, R. G. (1993). Initiation and termination of chromosome replication. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 507–528. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington DC: American Society for Microbiology.