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Reconstitution of the *B. subtilis* Replisome with 13 Proteins Including Two Distinct Replicases

Glenn M. Sanders,[1] H. Garry Dallmann,[1] and Charles S. McHenry[1,*]
[1]Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, USA
*Correspondence: charles.mchenry@colorado.edu
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SUMMARY

We have expressed and purified 13 proteins predicted to be required for *B. subtilis* DNA replication. When combined with a circular DNA template with a 5′ unpaired flap, these proteins reconstitute replication of both the leading and lagging strands at the physiological rate. Consistent with the in vivo requirement for two DNA polymerase III replicases for *B. subtilis* chromosomal replication, both PolC and DnaE are required for reconstitution of the replication fork in vitro. Leading strand synthesis requires PolC plus ten proteins; lagging strand synthesis additionally requires primase and DnaE. DnaE does not serve as the lagging strand replicase, like DNA polymerase δ in eukaryotes, but instead functions like eukaryotic DNA polymerase α, adding a stretch of deoxynucleotides to the RNA primer before handoff to PolC. Primase equilibrates with the fork prior to synthesis of each Okazaki fragment, and its concentration controls the frequency of initiation and Okazaki fragment size.

INTRODUCTION

For several decades, *E. coli* has provided the prototype for biochemical understanding of the replication of a cellular chromosome (Baker and Kornberg, 1991; Marians, 2000). Our mechanistic knowledge has been facilitated, and often led, by biochemical studies in complete systems encoded by bacteriophages λ, T4, T7, Φ29, and SV40 (Alberts, 1987; Benkovic et al., 2001; Collins et al., 1993; Nossal et al., 2007; Richardson, 1983; Salas et al., 1995; Stephens and McMacken, 1997; Waga and Stillman, 1994; Wang et al., 2000). The viral SV40-encoded origin recognition, helicase loader, and helicase activities of the multifunctional T-antigen have provided significant knowledge related to eukaryotic replication, yet no system is available for the reconstitution of cellular eukaryotic replication forks with purified proteins.

B. subtilis has provided a useful model system for understanding unique aspects of low-GC Gram-positive DNA replication, from both a genetic and biochemical perspective. Some features of *E. coli* replication are conserved: a PriA-mediated restart of stalled replication forks (Polard et al., 2002), a replicase comprising Pol III, β_2 and a DnaX complex (Bruck and O'Donnell, 2000), and a hexameric replicative helicase (Bruand et al., 1995). Important distinctions are also apparent that suggest that *E. coli* uses mechanisms that are not universally conserved, even among bacteria. *B. subtilis* appears to use two helicase loaders, like eukaryotic cells (Velten et al., 2003). *B. subtilis* also employs a different set of proteins that intervene between the PriA initiation protein and the helicase loader (DnaD in *B. subtilis* versus the unrelated PriB, PriC, and DnaT in *E. coli* [Bruand et al., 2001]). Particularly noteworthy is the requirement for two replicases, distinct homologs of DNA polymerase III, called PolC and DnaE (Dervyn et al., 2001). PolC differs from the *E. coli*-like DnaE in that it contains a different arrangement of conserved modules and contains the proofreading exonuclease as part of the same polypeptide chain (Hammond et al., 1991; Low et al., 1976).

Genetic studies have demonstrated that both PolC and DnaE are required for *B. subtilis* replication. Under conditions of DnaE deprivation, a small amount of leading strand DNA synthesis remained while lagging strand synthesis ceased. Based on this observation, it has been proposed that PolC might be the leading strand replicase and DnaE the lagging strand polymerase (Dervyn et al., 2001). Eukaryotes also use two polymerases, Pol ε and Pol δ, which constitute the core of the leading and lagging strand replicases, respectively (Kunkel and Burgers, 2008). A third eukaryotic polymerase, Pol α, is complexed with primase and functions to process the nascent RNA primer, adding a small number of deoxynucleotides before handing it off to the Pol δ replicase (Nethanel and Kaufmann, 1990).

Estimates have been made that *B. subtilis* and *E. coli* diverged over one billion years ago, even before plants and animals (Condon, 2003, and references therein). Thus, *B. subtilis* provides an opportunity to examine the extent to which *E. coli* serves as a uniform prototype for cellular replication. To enable this examination, we expressed all proteins predicted to be required for *B. subtilis* replication restart and used them to reconstitute a complete cellular replication fork system that will permit mechanistic-based probing of important differences, including the contribution of a second Pol III replicase.

RESULTS

Reconstitution of a *B. subtilis* Replication Fork

The collective work of laboratories that use genetic approaches suggested the requirement for 13 *B. subtilis* proteins to reconstitute a DNA replication fork. We expressed all 13 proteins, recombinantly, in *E. coli*. Proteins were expressed without appended tags and with the native sequence. This was done to avoid any functional perturbations that might result from unnatural sequences interfering with protein-protein interactions or other

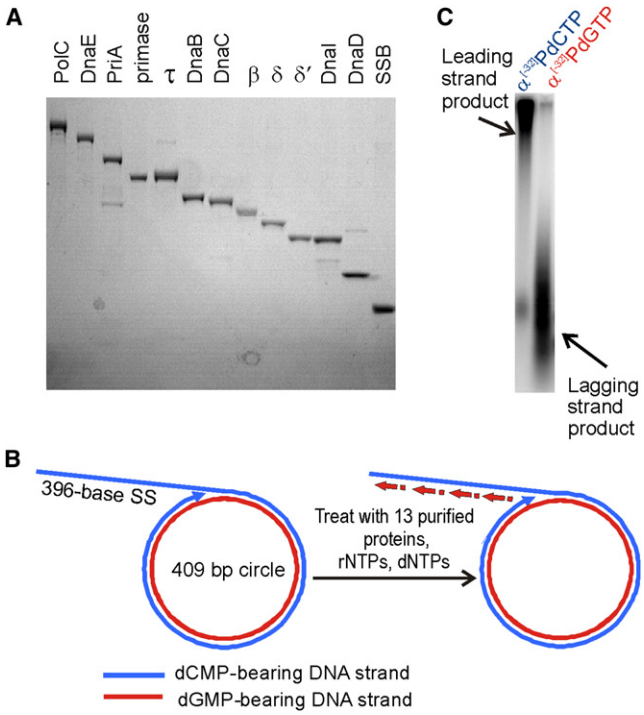

Figure 1. Thirteen Purified Proteins Are Sufficient for Reconstitution of *B. subtilis* Rolling Circle DNA Replication

(A) Thirteen purified recombinant *B. subtilis* DNA replication proteins were subjected to SDS-PAGE. Shown are PolC (166 kD), DnaE (125 kD), PriA (94.5 kD), primase (68.8 kD), τ (62.7 kD), DnaB (54.9 kD), DnaC (50.6 kD) β (42.1 kD), δ (42.1 kD), δ′ (40.5 kD), DnaI (36.1 kD), DnaD (28.8 kD), and SSB (18.8 kD). Approximately 700 ng of each purified protein was fractionated on a SDS-polyacrylamide gradient (4%–20%) gel and stained with Coomassie Brilliant Blue.

(B) Minicircle DNA replication template. The circular leading strand template is deficient in dCMP residues (G:C ratio 50:1). The flapped lagging strand template is deficient in dGMP residues. This permits quantification of leading and lagging strand synthesis by measuring radioactive dCMP and dGMP incorporation.

(C) Reconstitution of rolling circle synthesis. Addition of 13 purified *B. subtilis* DNA replication proteins to minicircle DNA in the presence of rNTPs, dNTPs, and α-[^{32}P] dCTP or α-[^{32}P] dGTP results in rolling circle DNA replication, as demonstrated by visualization of reaction products fractionated on a denaturing agarose gel. Very large DNA fragments, made visible in the presence of [^{32}P] dCTP, are derived from leading strand DNA synthesis. A parallel reaction that substitutes radiolabeled dGTP for dCTP renders visible the smaller Okazaki fragments from lagging strand DNA synthesis.

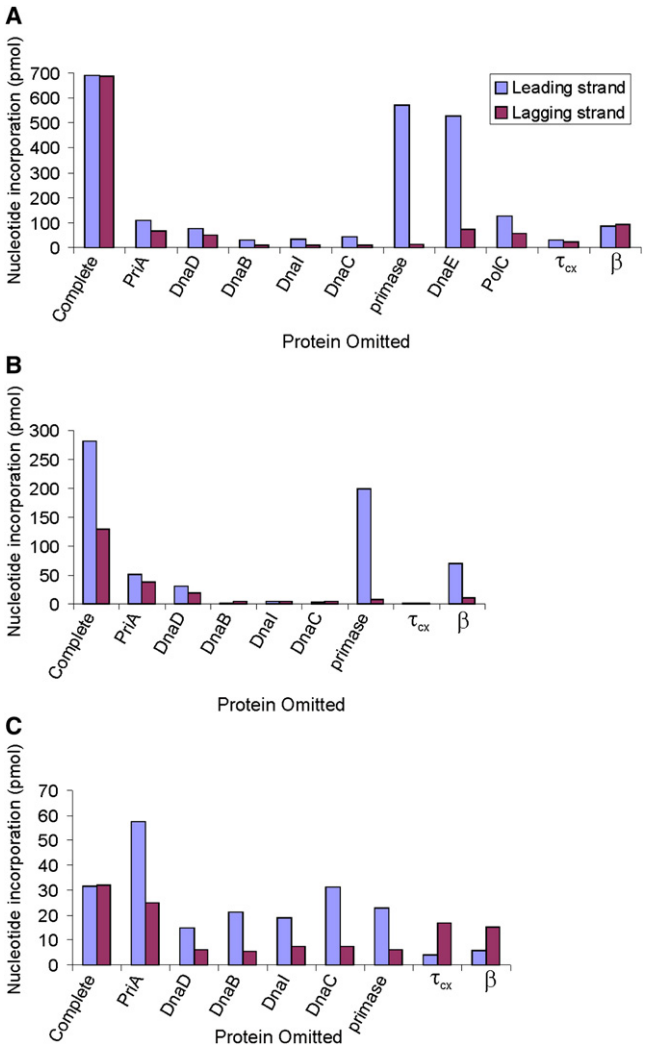

Figure 2. Protein Requirements for *B. subtilis* Rolling Circle DNA Replication

(A) Protein requirements in the presence of both *B. subtilis* DNA polymerase IIIs, PolC and DnaE. Leading and lagging strand syntheses were quantified by [^{3}H] dCMP (blue) and [^{3}H] dGMP (maroon) incorporation, respectively. The label τcx refers to a combination of the protein components of the τ complex: 12.5 nM τ tetramer, 12.5 nM δ monomer, and 12.5 nM δ′ monomer.

(B) Protein requirements in the absence of DnaE.

(C) Protein requirements in the absence of PolC.

functions. All 13 proteins were purified, initially using SDS-PAGE to guide the purifications, by standard chromatographic methods (Figure 1A).

We created a minicircular template, similar to those employed in other replication systems (Lee et al., 1998; Nossal et al., 2007; Yang et al., 2003) that had a strong (50:1) GC strand bias (Figure 1B). The template was designed so that labeled dGTP was incorporated, nearly exclusively, into the lagging strand product and labeled dCTP into the lagging strand product. Using methods similar to those developed by N. Nossal (Nossal et al., 2007), we made our minicircles larger than normally employed, to minimize steric issues. Upon addition of our 13 purified *B. subtilis* DNA replication proteins, robust leading and lagging strand synthesis were observed (Figure 1C). The lagging strand product was shorter (~1 Kb), corresponding to the standard size of Okazaki fragments, as observed in other systems (Chastain et al., 2000; Lee et al., 2002; Wu et al., 1992).

Thirteen Proteins Are Required for *B. subtilis* Replication Fork Synthesis In Vitro, Including Two Distinct DNA Polymerase IIIs

In assays where single proteins were omitted from an optimized replication fork assay, we observed that all 13 proteins are required for significant levels of lagging strand synthesis (Figure 2A). Primase and DnaE could be omitted from the assay

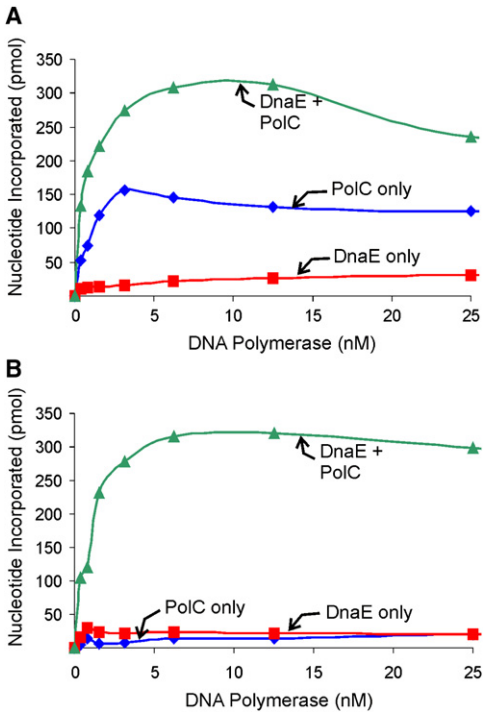

Figure 3. Both PolC and DnaE Are Required for Equivalent Leading and Lagging Strand DNA Synthesis

(A) Leading strand synthesis with varying DNA polymerase III concentrations. Reaction mixes containing primosomal proteins, primase, SSB, τ, δ, δ′, β, template DNA, rNTPs, and dNTPs were assembled with the indicated amounts of PolC, DnaE, or DnaE + PolC (equimolar) and incubated for 4 min prior to termination.

(B) Lagging strand synthesis with varying DNA polymerase III concentrations.

with maintenance of leading strand synthesis. This observation is consistent with the role of primase in generating primers for Okazaki fragment synthesis and the proposed role of DnaE in serving as the lagging strand replicase (Dervyn et al., 2001).

Since DnaE could be omitted and leading strand synthesis maintained, we re-examined protein dependencies in the presence of PolC as the only DNA polymerase. Again, we observed dependence on all proteins except primase (and DnaE) for leading strand synthesis (Figure 2B). The low levels of lagging strand synthesis observed remained dependent on all proteins.

In the absence of PolC, the DnaE-supported reaction lost specificity for proteins known to be required for replication in vivo. For example, we observed no leading strand requirement for the DnaC helicase, and the reaction is stimulated by omission of PriA (Figure 2C). Thus, the low level of replication observed in the absence of PolC is inauthentic, in violation of known genetic requirements.

To determine whether the requirement for one DNA polymerase could be overcome by increasing concentrations of the other, we varied polymerase concentration and quantified leading and lagging strand synthesis independently. Regardless of the PolC concentration, leading strand synthesis was reduced approximately 2-fold in the absence of DnaE (Figure 3A). In the presence of the full complement of proteins, leading strand synthesis could not be established in the absence of PolC, even at elevated DnaE concentration. Lagging strand synthesis was completely dependent upon both PolC and DnaE, regardless of the polymerase concentration used (Figure 3B).

Reconstituted Forks Progress at the Rate Observed In Vivo and Correlate with the Rate of PolC Holoenzyme Elongation

DNA replication forks progress at ~500 bp/s in *B. subtilis* at 30°C (Wang et al., 2007). We examined the elongation rate of reconstituted replication forks in the presence of PolC and/or DnaE (Figure 4). In the fully reconstituted system, forks progressed at 560 nt/s, consistent with the in vivo velocity. PolC alone supported a reaction at a nearly equivalent rate. However, the DnaE-supported reaction was very slow (25 nt/s). This latter observation is consistent with DnaE not functioning to elongate the leading strand at the replication fork, a requirement for generating the lagging strand template. We also measured the rate of

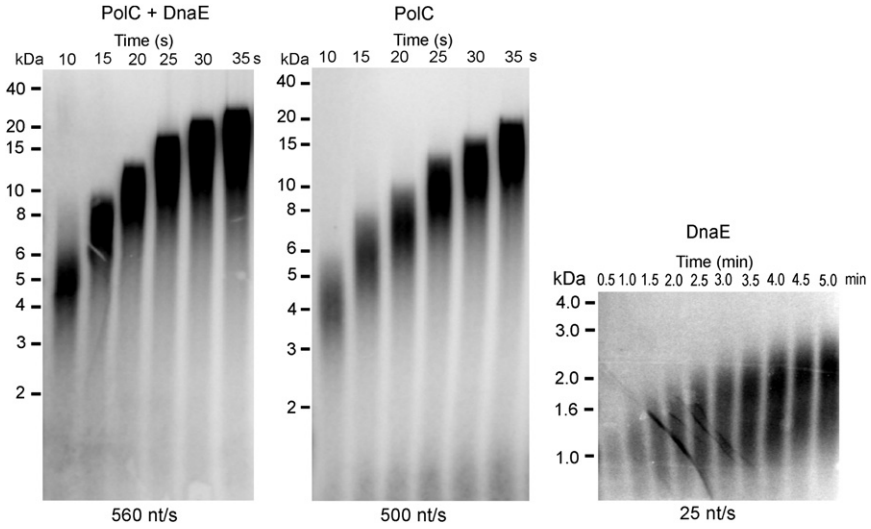

Figure 4. PolC Determines the Rate of Fork Progression

(Left) Extension rate of the complete rolling circle system in the presence of PolC and DnaE. Reactions were conducted as described for rolling circle extension rates in the Experimental Procedures. A rate of 560 nt/s was determined. (Center) Extension rate with PolC as the sole DNA polymerase. A rate of 500 nt/s was determined. (Right) Extension rate with DnaE as the sole DNA polymerase. A rate of 25 nt/s was determined.

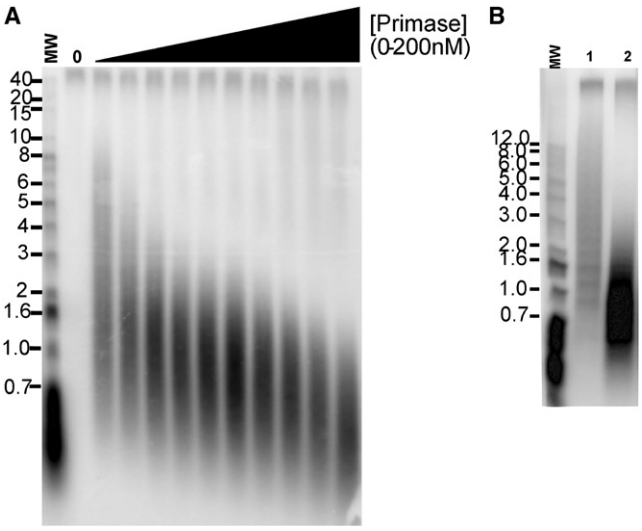

Figure 5. Primase Equilibrates with the Replication Fork Components, Making Primers that Are Only Efficiently Used in the Presence of DnaE

(A) Size of Okazaki fragments varies inversely proportionally to primase concentration. Rolling circle reactions were conducted as described in the Experimental Procedures, in the presence of α-[^{32}P] dGTP, and were quenched after 5 min. Primase was varied in a 2-fold dilution series, from 200 nM down to 0.4 nM.

(B) DnaE is required for efficient elongation of primers. Lane 2, reactions were conducted as described in (A) using 10 nM primase. Lane 1, reactions were conducted as in lane 2, except DnaE was omitted.

elongation by DnaE on long single-stranded templates that would more closely mimic the template for lagging strand synthesis. DnaE was very slow in the absence (13 nt/s) or presence (75 nt/s) of the τ complex and β_2 (Figure S2), consistent with previous observations (Bruck and O'Donnell, 2000).

Primase Cycles On and Off the Fork during Okazaki Fragment Synthesis

In the only other cellular system reconstituted at the replication fork level, *E. coli*, it has been found that DnaG primase cycles on and off the replication fork, through association with DnaB helicase, with the synthesis of each RNA primer for Okazaki fragment synthesis (Tougu and Marians, 1996; Wu et al., 1992). For this reason, the length of Okazaki fragments is inversely proportional to the DnaG primase concentration. Higher concentrations of primase lead to more frequent associations with helicase, leading to more frequent priming and Okazaki fragment formation. We also observe a decreasing Okazaki fragment size with increasing DnaG primase concentration in our *B. subtilis* system. Okazaki fragment length varies from ~1.6 kb at 0.4 nM primase down to 0.5 kb at 200 nM primase (Figure 5A).

DnaE, but Not PolC, Efficiently Uses RNA Primers

PolC does not work efficiently as the sole DNA polymerase. We specifically looked at the ability of PolC and DnaE to elongate primers during Okazaki fragment synthesis by monitoring [^{32}P]-dGTP incorporation (Figure 5B). Only DnaE could efficiently elongate RNA primers generated at the replication fork.

We next examined the relative abilities of PolC and DnaE to use RNA oligonucleotides for DNA synthesis on single-stranded model templates for Okazaki fragment initiation. We observed that both PolC and DnaE could efficiently elongate a DNA primer, but under the conditions used, only DnaE could elongate an RNA primer (Figure 6A).

To eliminate the potential for an artifact created by an RNA degradative contaminant in our PolC preparations, we performed two controls. In the first, we showed that mixing PolC with DnaE did not destroy the ability of DnaE to use an RNA primer (Figure 6A). In the second, we demonstrated that primer degradation did not occur during any of the PolC and/ or DnaE-containing reactions (Figure 6B).

In separate experiments, we used elevated levels of polymerase and dNTPs to see if we could force PolC to use an RNA primer (see Figure S3 available online). Both PolC and DnaE used DNA-primed templates with nearly equivalent efficiency (K_m for dNTPs of 16 and 18 μM, respectively). RNA primers were also elongated by DnaE efficiently (K_{mdNTP} = 13 μM). PolC, even at 2-fold higher concentration, was inert on an RNA primer until dNTP concentrations were elevated (>25 μM; Figure S3), supporting the notion that PolC does not efficiently elongate an RNA primer.

DnaE and PolC Cooperatively Extend Primers

The preceding experiments suggest that DnaE extends RNA primers initially and then hands them off to PolC for more extensive, rapid elongation. To test this, we used two assays, both RNA primed to force DnaE elongation prior to any potential action by PolC. For the first assay, to closely mimic the situation on the lagging strand of the replication fork, we set up a general priming system analogous to the one first developed with *E. coli* proteins (Arai and Kornberg, 1979). Like in the *E. coli* system, we used an SSB-coated long single-stranded template, loaded helicase using the requisite accessory proteins and generated primers by the reversible association of primase with helicase (Figures 7A and 7B). In this system, PolC and DnaE exhibited higher levels of synthesis than either polymerase alone or the total of their activities, indicating a synergistic effect, consistent with PolC rapidly elongating primers after an initial slow processing by DnaE (Figure 7A). PolC only showed significant stimulation after a kinetic lag (1.5 min under the conditions used) consistent with a limiting step preceding its action.

Additional support for a handoff between DnaE and PolC was gained by exploiting HBEMAU, a PolC-specific inhibitor developed by Brown, Wright, and colleagues that binds protein and primed DNA forming a dead-end ternary complex (Low et al., 1974; Tarantino et al., 1999). PolC alone cannot efficiently use RNA primers, but if it normally gains access to primers by a handoff mechanism after minimal extension by DnaE, addition of HBEMAU would be expected to inhibit the reaction markedly. Indeed, we observe such inhibition (Figure 7B). HBEMAU does not affect the reaction with DnaE by itself but nearly completely inhibits reactions that contain PolC, indicating PolC obtains access to primer termini from DnaE. Control experiments show

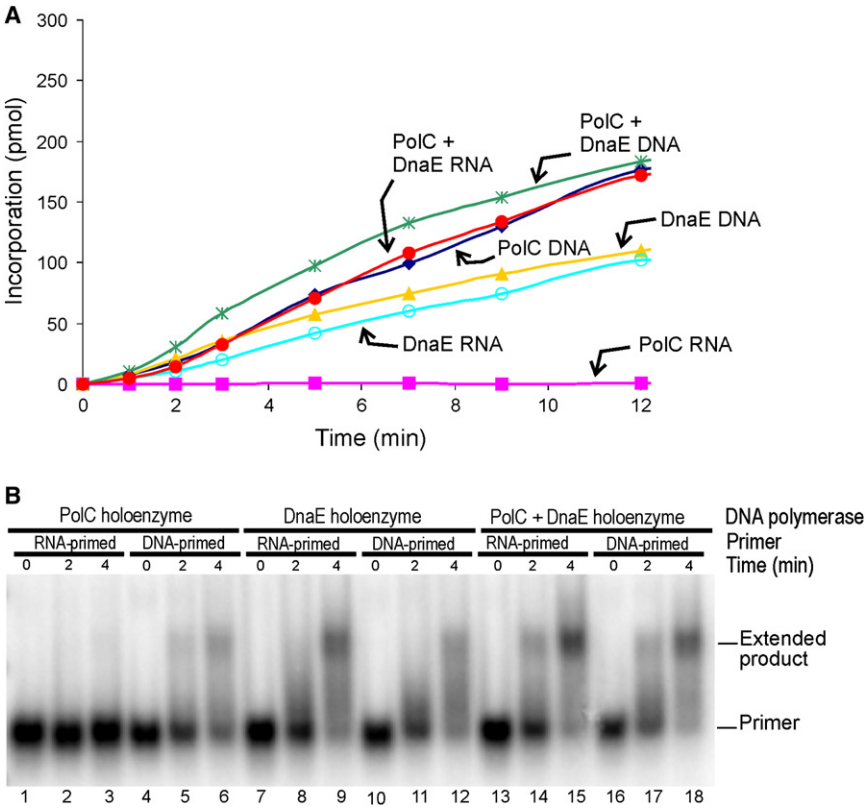

Figure 6. PolC Elongates DNA, but Not RNA Primers

(A) DNA synthesis using RNA or DNA primers. DNA or RNA primers were annealed to a single-stranded circular template and elongated as described in the Experimental Procedures, except 1 nM of the designated DNA polymerase was used.

(B) RNA primers remain intact in the presence of PolC. [^{32}P]-labeled primers annealed to a single-stranded circular template were treated with DNA polymerases and processed as described in the Experimental Procedures. Of DNA polymerase, 10 nM was used.

the same effect is observed, even when DnaE and accessory proteins are incubated with RNA-primed DNA before the addition of PolC (Figure S4).

In a second assay, we annealed synthetic RNA primers, again to mimic the initiation reaction on the lagging strand. We performed these assays at a constant level of PolC (if present) and varied DnaE for a set time. In spite of PolC exhibiting little activity on RNA primers by itself, it afforded significant stimulation in the presence of DnaE, especially at low DnaE levels (Figure 7C). PolC also stimulated reactions containing saturating levels of DnaE, strongly supporting a cooperative reaction where a handoff occurs between polymerases. As with the general priming assays, inclusion of the PolC-specific inhibitor HBEMAU resulted in nearly complete inhibition of the reaction, indicating PolC normally gains access to primer termini early in the Okazaki fragment reaction cycle after limited synthesis by DnaE and a handoff to PolC (Figure 7D).

DISCUSSION

Guided largely by genetics-based predictions, we have expressed 13 *B. subtilis* proteins in *E. coli* and found that, when

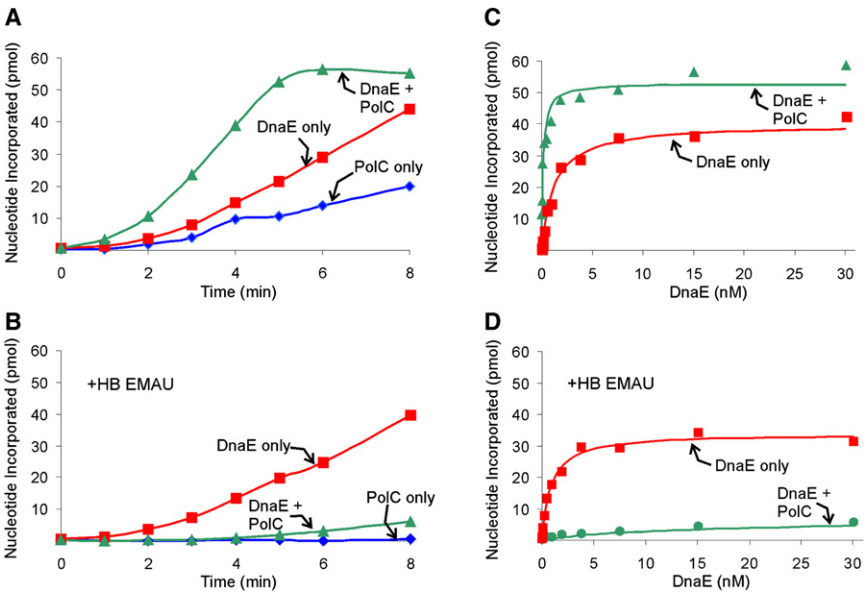

Figure 7. DnaE and PolC Cooperate in the Extension of a Primase-Synthesized Primer

(A) Reactions in (A) and (B) were performed as described in "General Priming" in the Experimental Procedures. (A) Extension of RNA primers was synthesized by in situ by primase in the presence of PolC, DnaE, or DnaE + PolC.

(B) Reactions were performed as above but also included 48 μM HBEMAU. Reactions in (C) and (D) were performed as described in "Primer Extension Assays" in the Experimental Procedures, except with 1.2 nM RNA primed template, 10 μM of each dNTP, 60 nM PolC, 60 nM β_2, 32 nM τ_4, 125 nM δ and δ', and 88 nM SSB_4. Reactions were conducted for 3 min at 30°C.

(C) Extension of synthetic RNA primers in the presence or absence of PolC at the specified concentration of DnaE.

(D) Reactions were performed as above but also included HBEMAU.

added together on a synthetic rolling circle template, they reconstitute a functional DNA replication fork that moves at the same rate observed in vivo. We observe synthesis of a long leading strand and discontinuous synthesis of Okazaki fragments of the predicted size on the lagging strand. In sharp contrast to the model derived from *E. coli*, synthesis is dependent upon two distinct DNA polymerase IIIs, PolC and DnaE. Lagging strand synthesis is dependent on the presence of primase and, as would be predicted, leading strand synthesis is not. We expect, in future studies guided by proteomic and functional biochemical approaches, that additional auxiliary proteins will be discovered. But for now, it appears that the 13 proteins already identified enable all of the major features required for rapid, processive replication fork progression.

This *B. subtilis* rolling circle replication fork system represents the second cellular system reconstituted from purified proteins to date. Although advanced knowledge is available in archaeal and eukaryotic systems, this feat has not been accomplished yet, perhaps due to missing components or complications imposed by complex regulation. The availability of this second cellular replication system from a divergent organism provides an opportunity to explore which features of the *E. coli*-based model are conserved and what variations can occur. As described in more detail in the introduction, many of the basic features of low-GC Gram-positive DNA replication are conserved, but important distinctions indicate that the applicability of the *E. coli* model is not universal.

Our studies establish that proteins predicted by partial DNA replication reactions and from genetic studies (see the Introduction) are sufficient to reconstitute a full replication fork reaction, initiated on a flapped rolling circle template. Our work demonstrates, as expected from the *E. coli* paradigm (Marians 2000), that primase is required exclusively for lagging strand synthesis and that it equilibrates with the replication fork between synthesis of successive Okazaki fragments. Decreasing primase concentration leads to less-frequent associations and longer Okazaki fragments.

This study supports the notion that PolC serves as the leading strand replicase. Rapid, efficient leading strand synthesis continues in the absence of DnaE. Consistent with previous predictions (Dervyn et al., 2001), lagging strand synthesis is dependent upon DnaE. Lagging strand synthesis is also dependent upon the presence of PolC, but that could be due to an indirect role: PolC is required to generate the template for lagging strand synthesis. However, certain characteristics of DnaE suggest it is not the sole lagging strand replicase and that its role more closely mimics that of Pol α in eukaryotes (Stillman 2008). First, DnaE is a very slow polymerase, elongating at a maximal rate of 75 nt/s under the experimental conditions used for reconstitution of replication forks. This is significantly slower than the in vivo rate of fork progression ($\sim$500 nt/s; Wang et al., 2007) and could not support lagging strand synthesis, at least in a coupled system. Second, DnaE, like Pol α, lacks an intrinsic proofreading nuclease and has been demonstrated to be error prone in vitro (Bruck et al., 2003; Le Chatelier et al., 2004), which is inconsistent with an extensive role in replication that would lead to unacceptable mutation rates on the lagging strand template.

DnaE has the ability to preferentially use RNA primers under the experimental conditions employed in our reconstituted fork assay. At low concentrations of polymerase and dNTPs that may mimic concentrations available to replication forks in vivo, we observe almost exclusive use of RNA primers by DnaE. In control experiments, the efficiency of priming by DNA primers with a sequence equivalent to the RNA primers is comparable between the two polymerases. A similar situation has recently been observed in a two-polymerase herpes virus replication reaction, where it was proposed that Pol α serves to process RNA primers, adding deoxynucleotides before handing off to a herpes-encoded DNA polymerase that cannot use RNA primers at physiological dNTP concentration (Cavanaugh and Kuchta, 2009).

Consistent with a handoff mechanism whereby DnaE initially extends an RNA primer followed by more extensive rapid elongation by PolC, we observe a synergistic effect if both polymerases are present simultaneously in reactions in which RNA primers are used. Blockage of DNA replication by the PolC-specific inhibitor HBEMAU provides additional evidence for a handoff mechanism whereby PolC gains access to 3′ termini of elongating strands early in the normal reaction. HBEMAU forms a ternary complex with primed DNA and PolC, arresting synthesis and denying access to other polymerases (Low et al., 1974; Tarantino et al., 1999). HBEMAU inhibits the PolC + DnaE reaction far below the level of DnaE alone. In eukaryotic systems, Pol α extends RNA primers a short distance, and, if Pol δ is present, a handoff occurs. In the absence of Pol δ, Pol α distributively reassociates and catalyzes further elongation, forming DNA products longer than it normally would (Murakami and Hurwitz, 1993; Nethanel and Kaufmann, 1990; Tsurimoto and Stillman, 1991; Waga and Stillman, 1994). The suppression of the DnaE-catalyzed synthesis in the presence of HBEMAU and PolC suggests a similar mechanism likely exists in *B. subtilis* where PolC normally gains access to 3′ termini early in the replication cycle.

The availability of a multiprotein Gram-positive replication system will permit detailed characterization of the unique interactions that lead to coupling of leading strand synthesis with a lagging strand reaction that involves two distinct Pol IIIs and will provide a platform for discovery and assessment of the contributions of auxiliary factors.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and Vent DNA polymerase (exo+) were purchased from New England Biolabs. Heparin Sepharose 4B, Q-Sepharose Fast Flow, SP-Sepharose Fast Flow, and butyl-Sepharose 4B were purchased from GE Biosciences. Hydroxyapatite (hypatite C) was purchased from Clarkson Chemical Co. N3-hydroxybutyl 6-(3′-ethyl-4′-methylanilino) uracil (HBEMAU) was a generous gift of Neal Brown and George Wright.

Buffers

Buffer H consisted of 40 mM HEPES-NaOH (pH 7.6), 10% v/v glycerol, 1 mM EDTA, 0.5 mM EGTA, and 1 mM dithiothreitol (DTT). Buffer T consisted of 40 mM Tris-HCl (pH 8.0), 10% v/v glycerol, 1 mM EDTA, 0.5 mM EGTA, and 1 mM DTT. Buffer I consisted of 50 mM imidazole (pH 6.8), 10% v/v glycerol, and 1 mM DTT. TE buffer consisted of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. QIAGEN buffer QC consisted of 1.0 M NaCl, 50 mM MOPS (pH 7.0),

and 15% isopropanol; and QIAGEN buffer QF consisted of 1.25 M NaCl, 50 mM Tris-Cl (pH 8.5), and 15% isopropanol. The designation ("prime") appended to a buffer name indicates omission of the metal chelators EDTA and EGTA. Buffer BsRC consisted of 40 mM Tris-acetate (pH 7.8), 12 mM magnesium acetate, 300 mM potassium glutamate, 3 μM $ZnSO_4$, 2% w/v polyethylene glycol (MW ~8000), 0.02% Pluronic F68, and 1 mM DTT. TAE gel buffer consisted of 40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA. TBE gel buffer consisted of 89 mM Tris, 89 mM boric acid, and 2 mM EDTA. Alkaline agarose gel buffer consisted of 30 mM NaOH and 0.5 mM EDTA.

Purification of *B. subtilis* DNA Replication Proteins

All proteins were expressed in *E. coli*, from either pET- or pA1-based vectors (Kim and McHenry, 1996a). Overexpression constructs encoded native *B. subtilis* 168 amino acid sequences without added tags. DnaI and DnaC were coexpressed from a single operon, following a report that such coexpression was required to generate soluble DnaC (Velten et al., 2003). Expression strains were grown in a 250 L fermentor in F-medium (Kim and McHenry, 1996b) supplemented with 1% w/v glucose and ampicillin (100 mg/L) at 37°C to OD_{600} = 1.0. IPTG was then added to 0.5 mM. Cells were harvested after 2 hr of induction. Harvested cells were suspended in an equal volume of a solution consisting of 50 mM Tris-HCl (pH 7.5) and 10% w/v sucrose. The resulting cell paste was frozen in liquid nitrogen and stored at −70°C.

PriA, DnaD, DnaB, DnaI, DnaC helicase, DnaG primase, DnaE, and SSB were monitored by SDS-PAGE after staining with Coomassie Brilliant Blue R250. Purification of PolC, τ, δ, δ′, and β was monitored by an activity assay to be published elsewhere. All proteins were assayed for single- and double-stranded endonuclease activity at 4-fold over the working concentrations specified for rolling circle replication reactions for 30 min at 37°C. The test substrates (200 ng per reaction) were supercoiled pBsRC3 and M13 Gori for the double-stranded and single-stranded endonuclease reactions, respectively. Protein preparations were judged to be endonuclease-free by the criterion that incubation resulted in no increase in the amount of nicked or linear forms appearing in samples fractionated on native agarose gels and stained with ethidium bromide. Cell lysates (Fraction I, Fr I) were prepared by the spermidine/lysozyme/heat method for all protein preparations (Cull and McHenry, 1995). All purification steps were performed at ~4°C. Proteins were precipitated from Fr I with the designated concentration of ammonium sulfate and stirred for 30 min. Precipitates were recovered by centrifugation for 30 min at 12,000 × g. Suspended ammonium sulfate precipitates are designated as Fr II.

PriA Fr II was prepared by addition of ammonium sulfate to 55% saturation to Fr I. The resulting pellet (derived from Fr I generated from 25 g cells) was suspended in buffer T, the conductivity was measured, and additional buffer T was added to a conductivity equivalent to that of buffer T containing 200 mM NaCl. This material was loaded onto a heparin-Sepharose 4B column (60 mL) equilibrated in the same buffer. The column was washed with five column volumes of buffer T containing 400 mM NaCl, then with two column volumes of buffer H′. PriA was step-eluted with four column volumes of buffer H′ containing 800 mM NaCl. Eluted PriA was loaded onto a hydroxyapatite column (20 mL) that had been equilibrated in buffer H′ containing 400 mM NaCl. The column was developed with a gradient of 0–200 mM potassium phosphate in buffer H′ containing 400 mM NaCl. PriA (20 mg) eluted at about 120 mM potassium phosphate. Peak fractions were pooled and dialyzed against buffer T containing 20% v/v glycerol, then aliquoted, frozen in liquid nitrogen, and stored at −70°C.

DnaD Fr II was prepared by addition of ammonium sulfate to 40% saturation to Fr I. The resulting pellet (from 100 g cells) was suspended in 40 mL buffer T containing 200 mM NaCl. Proteins that precipitate at low salt concentration (including DnaD) were recovered by overnight dialysis against buffer T containing 80 mM NaCl and centrifugation at 20,000 × g. The protein pellet was suspended in 10 mL buffer T containing 200 mM NaCl. A second round of low-salt precipitation was performed by addition of one volume of buffer T without added NaCl. Precipitated proteins were recovered by centrifugation at 20,000 × g and resuspended in 20 mL buffer T containing 200 mM NaCl. This material was loaded onto a heparin-Sepharose 4B column (20 mL) that had been equilibrated in the same buffer. A 20 column volume gradient to 1 M NaCl in buffer T was then run. DnaD eluted at about 450 mM NaCl. Peak fractions were pooled, then dialyzed against dry polyethylene glycol (MW 20,000) until the protein concentration was about 2 mg/mL. The concentrated protein (18 mg) was then dialyzed against buffer T containing 500 mM NaCl and 20% v/v glycerol. Aliquots were frozen in liquid nitrogen and stored at −70°C.

DnaB Fr II was prepared by addition of ammonium sulfate to 40% saturation to Fr I. The resulting pellet (from 100 g cells) was suspended in buffer T without added NaCl and the conductivity adjusted to that of buffer T containing 50 mM NaCl. This material was loaded onto a Q-Sepharose Fast Flow column (180 mL) that had been equilibrated in buffer T′ containing 50 mM NaCl. The column was developed with a 10 column volume gradient to 500 mM NaCl in buffer T′. DnaB eluted at about 300 mM NaCl. Peak fractions were pooled and dialyzed against buffer H containing 50 mM NaCl. This material was loaded onto a heparin-Sepharose 4B column (60 mL) that had been equilibrated in the same buffer. The column was developed with a 20 column volume gradient to 1 M NaCl in the buffer H. DnaB eluted at about 580 mM NaCl. Peak fractions were pooled and the conductivity adjusted with ammonium sulfate to that of buffer H containing 1M ammonium sulfate. This material was loaded onto a butyl-Sepharose 4B column (20 mL) that had been equilibrated in the same buffer. The column was developed with a 20 column volume gradient to buffer H without added salt. DnaB (60 mg) eluted at about 180 mM ammonium sulfate. Peak fractions were pooled, aliquoted, frozen in liquid nitrogen, and stored at −70°C.

DnaI and DnaC Fr II was prepared by addition of ammonium sulfate to 32.5% saturation to Fr I. The resulting pellet (from 100 g cells) (containing both DnaI and DnaC) was suspended in buffer T and the conductivity adjusted to that of buffer T containing 50 mM NaCl. This material was loaded onto a Q-Sepharose Fast Flow column (180 mL) that had been equilibrated in buffer T containing 50 mM NaCl. The column was developed with a 20 column volume gradient to 750 mM NaCl in buffer T. DnaI eluted at about 180 mM NaCl, while DnaC eluted at about 400 mM NaCl. DnaI- and DnaC-containing fractions (55 and 45 mg, respectively) were separately pooled. DnaC was aliquoted, frozen in liquid nitrogen, and stored at −70°C. DnaI was dialyzed against buffer T and loaded onto a heparin-Sepharose 4B column (60 mL) equilibrated in the same buffer. The column was washed in the same buffer, then developed with a 10 column volume gradient to 200 mM NaCl. DnaI eluted at about 50 mM NaCl. Peak fractions (35 mg) were pooled, aliquoted, frozen in liquid nitrogen, and stored at −70°C.

Primase (DnaG) Fr II was prepared by addition of ammonium sulfate to 40% saturation to Fr I. The resulting pellet (from 100 g cells) was suspended in 80 mL buffer T without added salt, then dialyzed against buffer T that contained 50 mM NaCl. This material was loaded onto a Q-Sepharose Fast Flow column (180 mL) that had been equilibrated in the same buffer. The column was developed with a 10 column volume gradient to 500 mM NaCl. Primase eluted at about 110 mM NaCl. Peak fractions were pooled, then H buffer without added salt was added until the conductivity of the pool was the same as that of H buffer containing 50 mM NaCl. This material was then loaded onto a heparin-Sepharose column (60 mL) that had been equilibrated in the same buffer. The column was developed with a 10 column volume gradient to 500 mM NaCl in H buffer. DnaG eluted at about 250 mM NaCl. Peak fractions were pooled and loaded directly onto a hydroxyapatite column (20 mL) that had been equilibrated in H′ buffer containing 200 mM NaCl. The column was developed with a 20 column volume gradient to 200 mM potassium phosphate in H′ buffer. DnaG eluted at about 90 mM potassium phosphate. Peak fractions (40 mg) were pooled and dialyzed into H buffer that contained 20% v/v glycerol and 50 mM NaCl. The dialyzed material was aliquoted, frozen in liquid nitrogen, and stored at −70°C.

DnaE Fr II was prepared by addition of ammonium sulfate to 45% saturation to Fr I. The resulting pellet (from 30 g cells) was suspended in buffer T and the conductivity adjusted to that of T buffer containing 75 mM NaCl. This material was loaded onto a Q-Sepharose Fast Flow column (60 mL) that had been equilibrated in the same buffer. The column was developed with a 20 column volume gradient to 500 mM NaCl in T buffer. DnaE eluted at about 220 mM NaCl. Peak fractions were pooled and diluted with H buffer to a conductivity equivalent to that of H buffer containing 80 mM NaCl. This material was loaded onto a heparin-Sepharose 4B column (5 mL) that had been equilibrated in the same buffer. The column was developed with a 20 column volume gradient to 600 mM NaCl in H buffer. DnaE (12 mg) eluted at about 350 mM NaCl. Peak

fractions were pooled, aliquoted, frozen in liquid nitrogen, and stored at −70°C without any further manipulation.

SSB Fr II was prepared by addition of ammonium sulfate to 40% saturation to Fr I. The resulting pellet (from 200 g cells) was suspended in buffer T and the conductivity adjusted to that of T buffer containing 150 mM NaCl. This material was loaded onto a column of Blue-dextran (400 mL), then washed successively with T′ buffers containing 150 mM, 2 M, and 4 M NaCl. SSB eluted in the 2 and 4 M washes. Peak fractions were pooled and loaded onto a hydroxyapatite column (60 mL) that had been equilibrated in imidazole buffer containing 200 mM NaCl. Proteins were step eluted with imidazole buffer containing 70 mM potassium phosphate. Peak fractions (160 mg) were pooled and dialyzed against dry polyethylene glycol to a concentration of 2 mg/mL. The concentrated material was aliquoted, frozen in liquid nitrogen, and stored at −70°C.

Rolling Circle Assays

Reaction conditions for fixed-time assays consisted of 5 nM PriA monomer; 25 nM DnaD tetramer; 50 nM DnaB tetramer; 20 nM DnaI hexamer; 50 nM DnaC hexamer; 6 nM primase monomer; 10 nM DnaE monomer; 10 nM PolC monomer; 12.5 nM τ_4; 12.5 nM δ; 12.5 nM δ'; 12 nM β_2; 6.25 μg/mL SSB_4 (84 nM); 5 nM DNA template; 250 μM ATP; 100 μM CTP, GTP, and UTP; 25 μM dNTPs; and 0.2 μCi/reaction [^{3}H] dCTP or [^{3}H] dGTP, all in 25 μl of BsRC buffer. Reaction mixes were incubated at 37°C. Typically, an enzyme mix consisting of all proteins except SSB in buffer BsRC was generated, split, and added to two substrate mixes composed of template DNA, rNTPs and dNTPs, SSB, and [^{3}H]-dCTP (for measurement of leading strand synthesis) or [^{3}H] dGTP (for measurement of lagging strand synthesis), all in buffer BsRC. α-^{32}P-radiolabeled deoxynucleoside triphosphates (2000 cpm/pmol) were substituted for tritium-labeled deoxyribonucleoside triphosphates in reactions that were to be analyzed by gel electrophoresis.

Assays in which extension rates were to be measured were synchronized by assembling reaction mixes from which dCTP and dGTP were omitted. After a 5 min preincubation, dCTP, dGTP, and α-[^{32}P] dCTP (2000 cpm/pmol) were added. Aliquots were removed at the indicated times and added to an equal volume of a stop mix composed of 40 mM Tris-HCl (pH 8.0), 0.2% SDS, 100 mM EDTA, and 50 μg/mL Proteinase K. Samples were treated for 20 min at 37°C, then brought to 50 mM NaOH, 5% v/v glycerol, and 0.05% bromphenol blue. Samples were fractionated on alkaline 0.45% agarose gels for approximately 1 hr at 225 V. Gels were fixed in 7% trichloroacetic acid, dried, autoradiographed on storage phosphor screens, and analyzed with Molecular Dynamics Phosphorimager software. The MW of the longest leading strand reaction products in each (considered as the leading edge of the nucleic acid front) were determined graphically, as a function of their mobilities (Rf) on the above gels, using radiolabeled DNA size standards to plot a standard curve of log MW versus Rf. MW of reaction products was then used to calculate extension rates in nucleotides per second or nucleotides per minute.

Primer Extension Assays

Templates were prepared by combining 20 pmol single-stranded M13 Gori DNA with 100 pmol 30-mer RNA or DNA primers (5′-GAACGGTGTACAGACCAGGCGCATAGGCTG) in a buffer consisting of 50 mM HEPES (pH 7.8), 100 mM KCl, and 1 mM EDTA, heating to 80°C, and slow cooling to room temperature. Reaction mixes consisted of 5 nM RNA- or DNA-primed template; 0.5 nM PolC, 0.5 nM DnaE, or 0.5 nM PolC plus 0.5 nM DnaE; 12 nM β_2 6.25 nM τ tetramer, 6.25 nM δ monomer, and 6.25 nM δ' monomer; 40 nM dATP, 40 nM dGTP, and 40 nM dCTP; 18.8 nM [^{3}H]-dTTP (specific activity 100 cpm/pmol); and 250 μM ATP in buffer BsRC. The reaction was initiated by the addition of a substrate mix containing DNA template, ATP, and dNTPs to an enzyme mix containing DNA polymerase. Incubation was at 37°C. Aliquots (25 μL) were withdrawn at the indicated times and added to a stop mix consisting of 50 μL 10% TCA and 25 μL 0.2 M sodium pyrophosphate. TCA-precipitated reaction products were processed and quantified by scintillation counting in the manner described above.

Templates for the experiment shown in Figure 6B were prepared by 5′ end-labeling primers described in the preceding paragraph using γ-[^{32}P] ATP and T4 polynucleotide kinase, and annealing 10 pmol radiolabeled primer to 10 pmol M13 Gori. Reaction mixes consisted of 5 nM DNA template; 10 nM PolC, 10 nM DnaE, or 10 nM PolC plus 10 nM DnaE in buffer BsRC; 12 nM β_2; 6.25 nM τ tetramer, 6.25 nM δ monomer, and 6.25 nM δ' monomer; 25 μM dNTPs; and 250 μM ATP. Reactions were initiated by the addition of a substrate mix consisting of DNA template and ATP in buffer BsRC to an enzyme mix consisting of DNA polymerase. Incubation was at 37°C. Aliquots (25 μL) were withdrawn at the indicated times and added to 25 μL of a stop mix consisting of 100 mM EDTA, 0.2% w/v SDS, and 50 μg/mL Proteinase K. Reactions were treated for 20 min at 37°C, then 10 μL of a mix consisting of 50% v/v glycerol and 0.05% bromphenol blue was added. Samples were fractionated on 0.8% native agarose gels in TAE buffer, fixed in TCA, dried, and then autoradiographed.

General Priming Assays

B. subtilis general priming reactions consisted of 5 nM single-stranded mp18 DNA template; 20 nM PolC (when present); 4 nM DnaE (when present); 24 nM β_2; 12.5 nM τ_4; 12.5 nM δ; 12.5 nM δ'; 50 nM $DnaI_6$; 50 nM $DnaC_6$; 25 nM $DnaB_6$; 25 nM primase; 10 μM each of dATP, dGTP, dCTP, and [^{3}H]-dTTP (specific activity 176 cpm/pmol total nucleotide); 250 μM ATP; and 100 μM each of GTP, CTP, and UTP in buffer BsRC. The reaction was initiated by the addition of a substrate mix containing DNA template, ATP, and dNTPs to an enzyme mix. Incubation was at 37°C. Aliquots (25 μL) were withdrawn at the indicated times and incorporated nucleotide quantified as described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2009.12.025.

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Supplemental Information

Reconstitution of the *B. subtilis* Replisome with 13 Proteins Including Two Distinct Replicases

Glenn M. Sanders, H. Garry Dallmann, and Charles S. McHenry

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA templates

M13 Gori was prepared by PEG precipitation and SDS extraction (Johanson et al., 1984; Yamamoto et al., 1970). Single-stranded ΦX virion DNA was purchased from New England Biolabs. Minicircle DNA was generated from plasmid pBsRC3, which was created by chemical synthesis of an oligonucleotide that was inserted into the *Eco RI* site of pUC19-based vector pUCminusMCS. The sequence of the synthetic DNA insert is:
GAATTCGAGTCTCCTCACTCTCTTCCCCTCCATACCCTTCCTCCATCTATACCACCCTACTCT
CCTTTCCTCATTATTCCTCCTATTATCTTCTCCTCTTCTCTCCTTCTTCTATATTTCCCAAATCT
ATCATCATTCACTCTCATCCCCTCTTCCTTCACTCCCATTCTATTCTACTCCTTTCCCTTTCCA
TATCCCCTCCACCCCCCTTCCTCCCCTCTTTCAATCTTATCCCCAATCATAAAATTATCTCAAT
TATATTCTCCTTCCATACCCCCTATCATCCTCATCCCTATCACCCCCTACTCACCCAATACTC
CCTACTCATCTCATATATCCTTATCCTCTCCTCACCTCTCCCTCCTCTATCTCCCCCCCCTCAC
ACTCATTTCTCATTCCACTCCCCCTCAGAATTC.

Purified plasmid DNA (16 mg, 1mg/mL) was treated overnight with EcoRI restriction enzyme (4000 units) to release the 409 base-pair cassette. EDTA was added to 50 mM final concentration, and the restriction enzyme was heat inactivated (65 ^{o}C, 40 min). The solution was brought to 5% w/v polyethylene glycol (nominal MW 8000), 0.5 M NaCl, and 0.5 mg/mL plasmid DNA. The mixture was incubated for 48 hr at room temperature, and centrifuged for 1 hr at 4500 x g at room temperature. The supernatant, bearing the 409 base-pair DNA fragment, was carefully decanted. The supernatant (32 mL) was dialyzed against TE buffer overnight to remove NaCl and EDTA. The dialysate was then adjusted

to the following final concentrations: 2.5 μg/mL DNA, 25 mM Tris-acetate (pH 8.0), 10 mM magnesium acetate, 25 μg/mL BSA, 1 mM ATP, and 150 NEB units/mL T4 DNA ligase. The solution was incubated overnight at 16 ^{o}C. A portion was precipitated and the circularization efficiency determined by native agarose gel electrophoresis in TBE buffer in the presence of 0.5 μg/mL ethidium bromide. Circularization was >90% complete. The solution (800 μg DNA) was adjusted to 0.5 M NaCl loaded onto a single Qiagen Tip 500 column. The column was washed with Qiagen Buffer QC and the DNA eluted with Qiagen Buffer QF. DNA was precipitated by the addition of 0.7 volumes of isopropanol, washed with 70% ethanol, and suspended in TE buffer. The purified circular DNA was adjusted to a concentration of 1 mg/mL and 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The DNA was site-specifically nicked with N.BstNB1 nicking enzyme (2 units of enzyme/μg DNA) for 2 hr at 55 ^{o}C. After digestion, the enzyme was heat-inactivated via treatment at 80 ^{o}C for 30 min. Nicking efficiency was assessed by agarose gel electrophoresis as specified above. Nicking was >90% complete. The solution was brought to 100 μg/mL DNA, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 μM dATP, 200 μM dCTP, and 200 μM dTTP. Exonuclease-proficient Vent DNA polymerase (0.5 units/μg DNA) was added, and the mix was incubated at 75 ^{o}C for 40 min. EDTA was added to a final concentration of 25 mM, and the mixture was extracted twice with one volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was precipitated with ethanol, washed once with 70% ethanol, and suspended in TE buffer at a final concentration of 1 μM (0.41 mg/mL). Tailing efficiency was monitored by agarose gel electrophoresis as specified above. Tailing was >90% complete (Figure S1). Overall yields were, on a molar basis, 30-35% relative to starting plasmid.

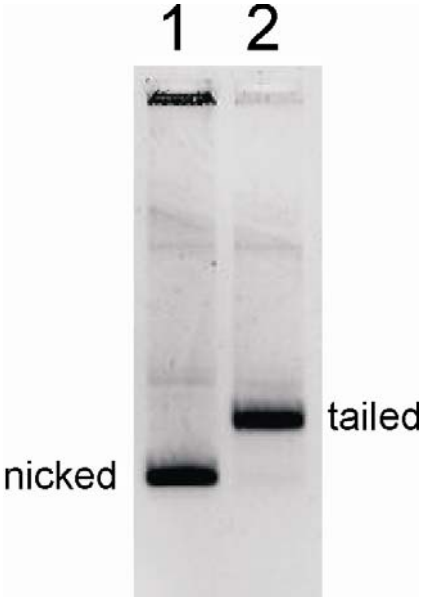

Figure S1. The Tailing Efficiency of the *B. subtilis* Midi-circle DNA Template.

Mini-circle DNA was excised from plasmid DNA, purified, circularized, nicked, and tailed as described under *Experimental Procedures*. 100 ng of the circular DNA from a nicking reaction (lane 1) and 100 ng of the midi-circle DNA from the ensuing tailing reaction (lane 2) were fractionated on a 1.2% native agarose gel and stained with ethidium bromide. Greater than 90% of the material from the nicking reaction exhibits reduced mobility after the tailing reaction.

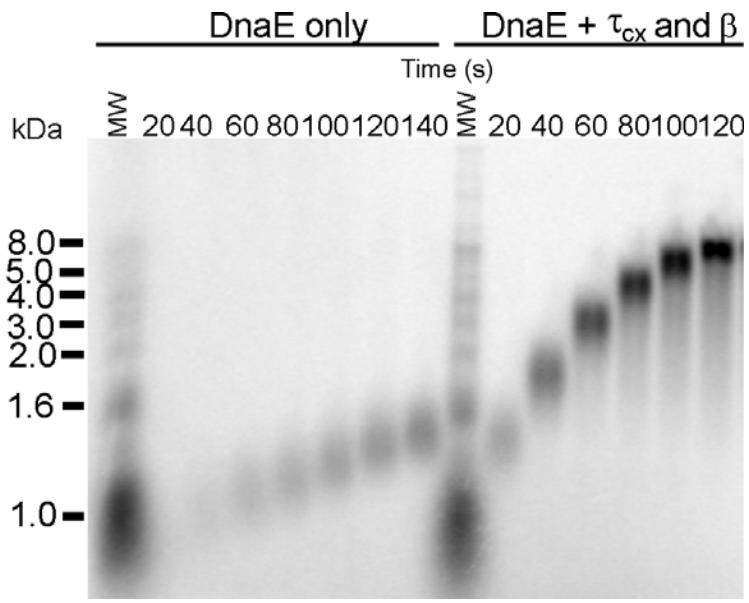

Figure S2. *B. subtilis* DnaE is Stimulated by the τ Complex and β .

The experiment reported on the left half of the gel was performed with primed single-stranded DNA template and DnaE. The measured rate of extension was 12.5 nt/s. The DnaE concentration was 10 nM. The experiment in the right panel was performed as above with the addition of 12.5 nM τ tetramer, 12.5 nM δ monomer, 12.5 nM δ' monomer, and 12 nM β_2. The measured rate of extension was 75 nt/s. Reactions were synchronized by first incubating all enzyme components for 5 min with template in the absence of dCTP and dGTP. Reactions were initiated by addition of dGTP and [^{32}P] dCTP (2000 cpm/pmol). Otherwise, reactions were conducted as described for Primer Extension Assays under *Experimental Procedures*.

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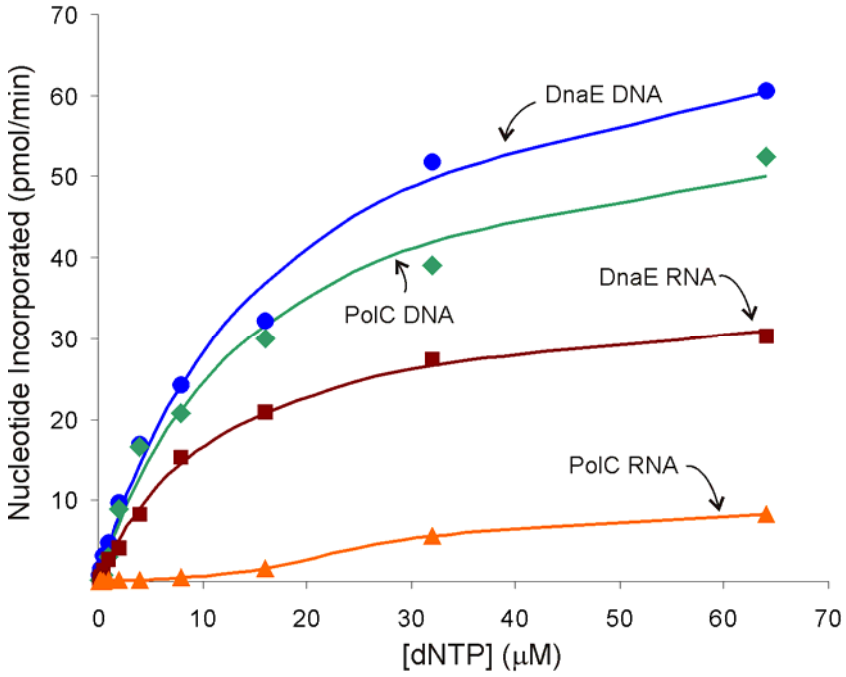

Figure S3. Primer Utilization by PolC and DnaE.

Reaction mixes contained 250 pmoles (total nucleotide) RNA- or DNA-primed template as described under *Experimental Procedures, Primer Extension Assays* except that reactions contained 5 nM PolC (exception listed below), 12 nM DnaE, 60 nM β_2, 32 nM τ_4, 125 nM δ and δ' monomer, 88 nM SSB_4, and 250 μM ATP. Deoxynucleotides were titrated with concentrations varying from 0-64 μM dATP, dCTP, dGTP, and [^{3}H]-dTTP (specific activity 280 cpm/pmol total nucleotide). The reaction with RNA-primed template and PolC used elevated (20 nM) levels of PolC to increase the efficiency of the reaction. Incubation was at 30 ˚C for 2 min, except for the RNA-primed reaction with PolC that was incubated for 4 min to increase synthesis proportionately. Data was fit to the Michaelis-Menten equation ($V= (V_{max} \times S)/ (S + K_m)$) using nonlinear least squares fitting to K_m values. The labels indicate the combination of polymerase and primer.

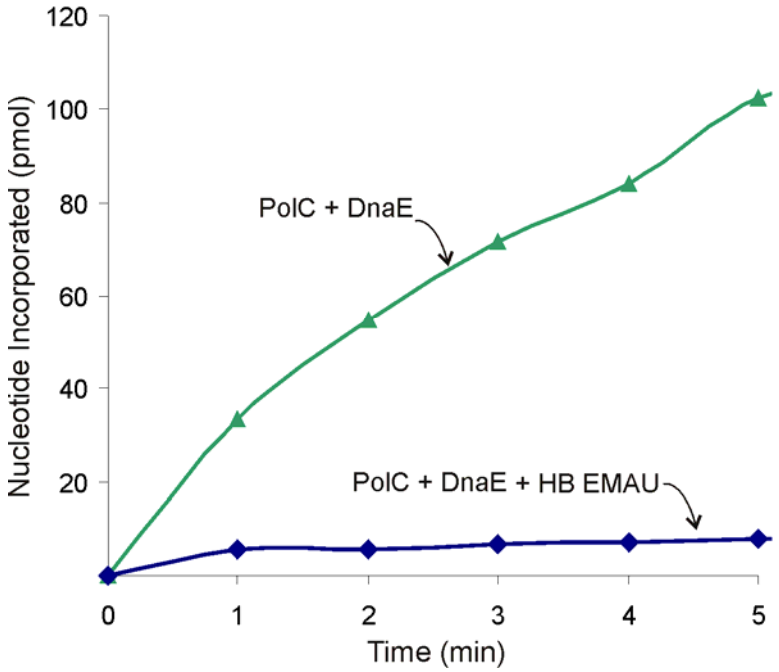

Figure S4. HBEMAU Inhibits Primer Elongation Even if DnaE and All Accessory Proteins are Pre-Incubated with Primed Template before Addition of PolC

Reactions were conducted as described under *Primer Extension Assays* in *Experimental Procedures* except DnaE (5 nM final) was preincubated with primed DNA in the presence of 24 nM β_2, 12,5 nM τ_4, δ and δ', 10 μM dATP, dCTP, dGTP and 250 μM ATP at 37 ^{o}C for 3 min before the addition of [^{3}H] dTTP (10 μM) and PolC (5 nM final). Where specified, HBEMAU was 48 μM.

SUPPLEMENTAL REFERENCES

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