Supplementary Data

for the manuscript

Bacillus subtilis DNA polymerases, PolC and DnaE, are required for both leading and lagging strand synthesis in SPP1 origin-dependent DNA replication

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This supplementary material contains:
Supplementary methods
Supplementary text
Supplementary references
Supplementary Figures (S1 to S8)

Supplementary methods: Protein-protein interaction assays

PolC-DnaG interaction. Purified N-terminal His-tagged DnaG and nontagged PolC (3 μg each) were incubated for 15 min at 4 ºC in 100 μl of binding buffer (25 mM Tris-HCl pH 7.5, 2 mM DTT, 5% glycerol, 150 mM NaCl), and then the proteins mixtures were incubated with 20 μl of pre-equilibrated nickel-magnetic beads (Invitrogen) for 10 min at 4ºC with gentle mixing. After washing the beads once with 200 μl of binding buffer, the bound proteins were eluted with 30 μl of SDS-PAGE sample loading buffer. The flow trough and wash fractions were concentrated by 20% TCA precipitation. All fractions were analyzed by SDS-PAGE and Coomassie blue staining.

Coprecipitation of SsbA or G*36*P with DnaE. Coprecipitation was analyzed by ammonium sulfate precipitation using a method described in (1) with minor modifications. SSBs precipitate at low ammonium

sulfate concentrations, whereas non-interacting proteins remain in the supernatant. Purified DnaE was incubated with G*36*P or SsbA (3 μg each) for 15 min at 4 ºC in 40 μl of binding buffer (25 mM Tris-HCl pH 7.5, 2 mM DTT, 5% glycerol, 150 mM NaCl), and then 140 μl of precipitation buffer (binding buffer additionally containing 2.3 M ammonium sulfate) was added to the protein mixtures to give an ammonium sulfate concentration of 1.6 M. After 10 min at 4ºC, samples were centrifuged for 5 min at 14000 rpm in a cooled centrifuge. The supernatant was removed to a fresh tube and the pellet was then washed with 200 μl of precipitation buffer followed by an identical centrifugation run. The final pellet was resuspended in 30 μl of SDS-PAGE sample loading buffer. The supernatant and wash fractions were precipitated with 20% TCA to concentrate them. All fractions were analyzed by electrophoresis in 12% SDS–PAGE gels. Following electrophoresis, gels were stained with Coomassie brilliant blue.

Supplementary text: *in vitro ori*L dependent DNA replication is unidirectional and proceeds in some molecules clockwise and in others counterclockwise

Previously it has been shown that *in vitro* DNA replication with an *E. coli oriC*-plasmid template was bidirectional, and this required modulation of topology by DNA gyrase (2). In the θ-type *ori*L-SPP1 system, a minimal concentration of DNA gyrase was needed to achieve complete DNA synthesis, and only unit-length products accumulated at the different DNA gyrase concentrations tested (Supplementary Figure S2). No leading strand products expected from a bidirectional replication (i.e., with half the size of the pCB163 plasmid template, because two opposite replication forks progress until they meet) were detected in the alkaline gels, even at high DNA gyrase concentrations. To confirm that *ori*L dependent DNA replication is unidirectional in our *in vitro* system, and to analyze the direction of DNA replication, we incubated the replications reactions, set up with plasmid pCB163 as the DNA template, in the absence of DNA gyrase for 4 min. This allowed DNA replication to start and accumulated early replication intermediates (ERIs). After that, a restriction enzyme (*Pst*I or *Eco*RV) was added to remove the torsional stress, and reactions continued for another 15 min to run-off replication up to the restriction site. The results were analyzed by denaturing alkaline gel electrophoresis and autoradiography (Supplementary Figure S4A and S4B). In the run-off reactions performed with the *Pst*I enzyme two bands of 4.6 and 2.3 Kb were detected, confirming the start of DNA replication from the *ori*L region. These products could correspond to a bidirectional replication, or

alternatively to an unidirectional replication, which moves clockwise in some plasmids and counterclockwise in others, to yield leading strand products of 4.6 and 2.3 kb respectively (Supplementary Figure S4A and S4B). A mixed population of directions was observed in phage λ *in vitro* unidirectional replication (3). In the *Eco*RV run-off reactions, we detected two leading strand products of 6.1 and 0.8 kb. In this case, the restriction site is located between the *ori*L site and the position of replication stalling by accumulation of torsional stress (ERI, see figure S4A). If DNA replication initiated bidirectionally, DNA products shorter than 6 kb should be expected after digestion with the *Eco*RV enzyme, because two forks coming from opposite directions stop when converging in one position, and this was not observed, suggesting that in our *ori*L system, DNA replication is unidirectional and moves in some plasmid molecules clockwise and in others counterclockwise.

To confirm SPP1 unidirectional DNA replication, we did a last experiment. We incubated the reactions in the absence of DNA gyrase for 4 min, to allow ERI formation. Then replication reactions continued for another 15 min in the presence of *E. coli* DNA Polymerase I and T4 DNA ligase (NEB Biolabs, 1U each). If DNA replication is bidirectional, the addition of DNA Pol I and DNA ligase should result in new bands, corresponding to the ligation of one leading strand ERI with the Okazaki fragments synthesized on the same template molecule by the other replisome, which moves in the opposite direction (i.e, we expect bands around 3 kb, which is the sum of the size of the two ERIs observed). When these replication products were analyzed in a denaturating gel, we observed the disappearance of the Okazaki fragments, which were efficiently completed and ligated, so that they were converted into products with the size of the leading strands ERIs. A ~3 kb band, corresponding to the ligation of a leading strand ERI with the Okazaki fragments synthesized by the other replication fork, was almost not detected, indicating that in most of the molecules DNA replication is unidirectional (Supplementary Figure S4C).

Supplementary References

1. Buss, J.A., Kimura, Y. and Bianco, P.R. (2008) RecG interacts directly with SSB: implications for stalled replication fork regression. *Nucleic Acids Res*, **36**, 7029-7042.
2. Smelkova, N. & Marians, K. J. (2001) Timely release of both replication forks from *oriC* requires modulation of origin topology. *J. Biol. Chem.* **276**, 39186-39191.
3. Mensa-Wilmot, K. *et al.* (1989) Reconstitution of a nine-protein system that initiates bacteriophage lambda DNA replication. *J. Biol. Chem.* **264**, 2853-2861.

SUPPLEMENTARY FIGURES

Supplementary Figure S1

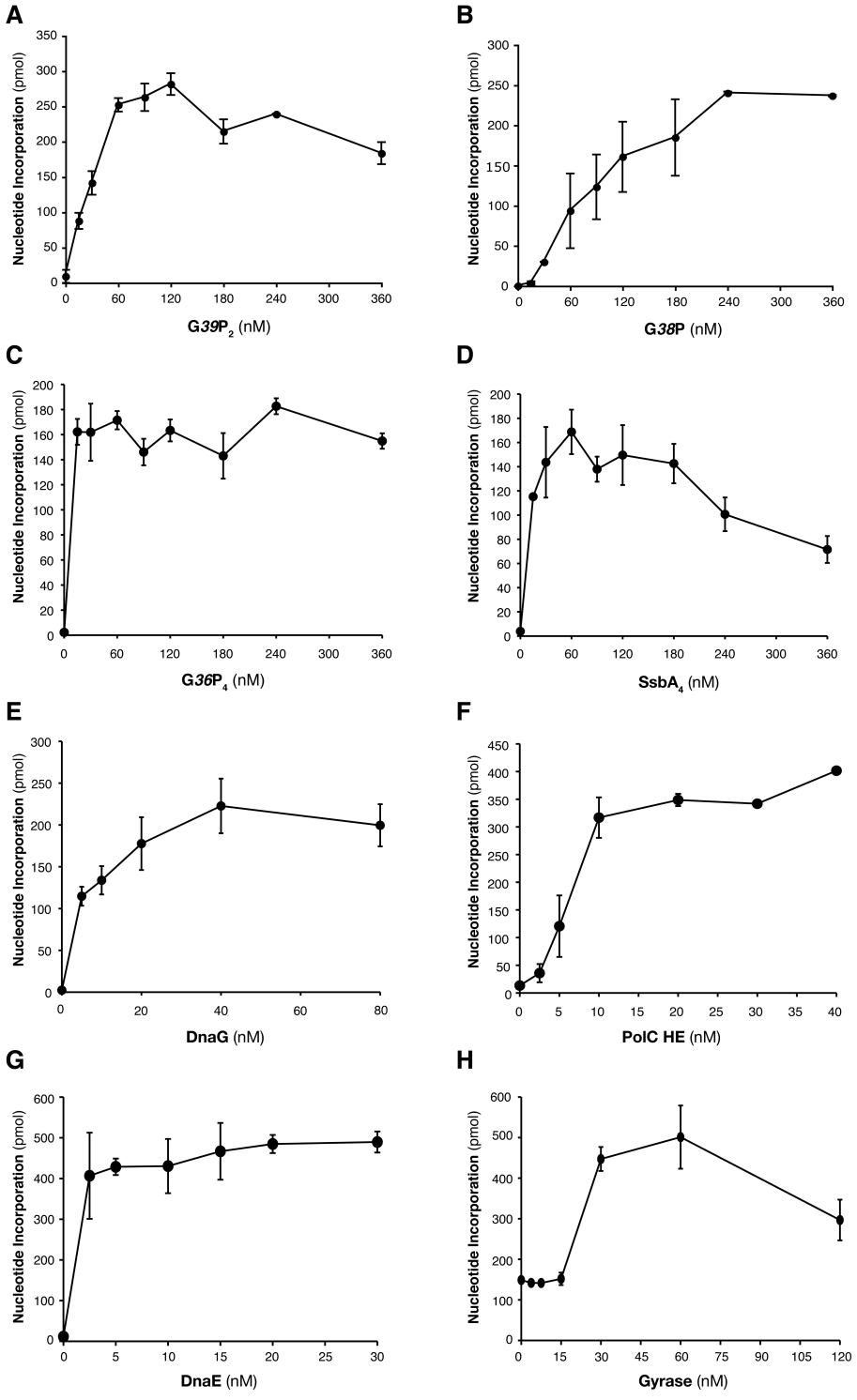

Figure S1. Optimization of *in vitro ori*L-dependent θ-type DNA replication. The reactions were performed with plasmid pCB163 as the DNA template for 15 min at 37ºC. Measurement of DNA synthesis was performed as described in Material and Methods. All titrations were carried out in the presence of optimal concentrations of the other protein components. Values are the mean of three independent experiments.

Supplementary Figure S2

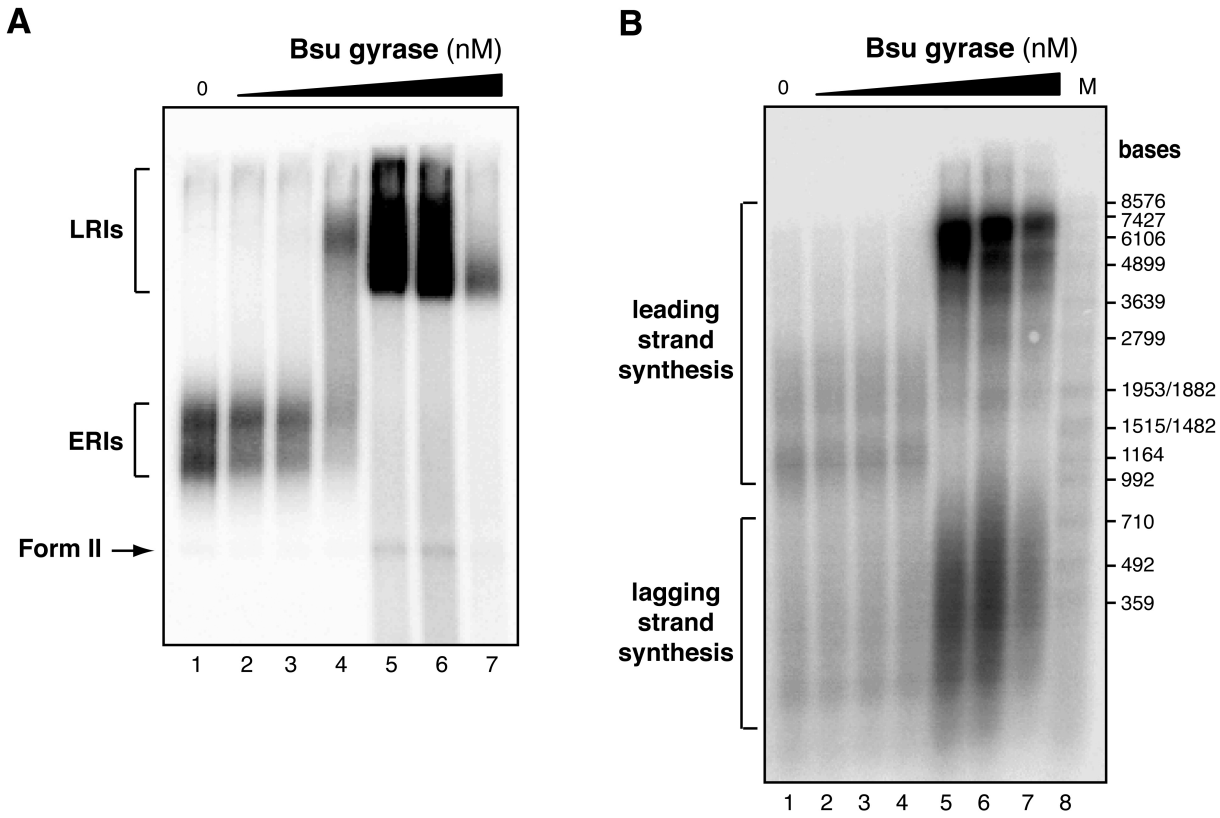

Figure S2. DNA replication products obtained varying *B. subtilis* DNA gyrase concentrations. Standard *ori*L-dependent θ-type DNA replication reactions, with plasmid pCB163 as the DNA template, were performed varying both GyrA and GyrB in a 2-fold dilution series, from 120 nM down to 3.75 nM, or in the absence of DNA gyrase. The radioactive products were analyzed in a native agarose gel **(A)** or in a denaturing alkaline agarose gel **(B)** followed by autoradiography. Abbreviations: LRIs, late replication intermediates; ERIs, early replication intermediates.

Supplementary Figure S3

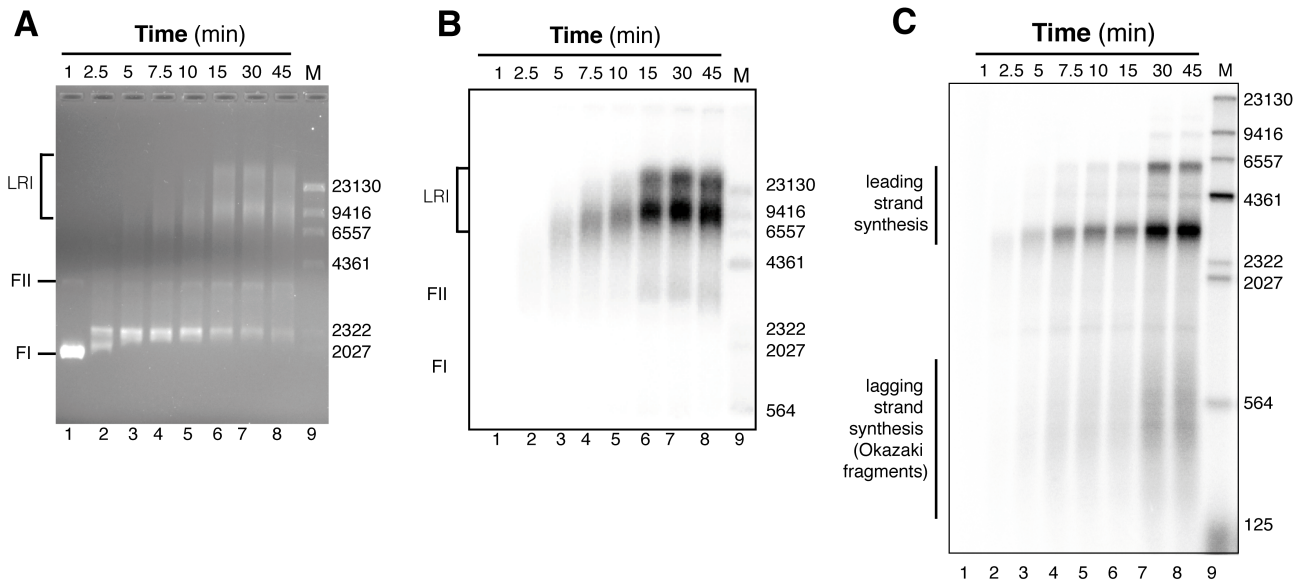

Figure S3. Time course analysis of the DNA products generated in a replication reaction performed with plasmid pBT430 as template DNA. (**A**) One half of the replication products was analyzed in a native agarose gel stained with ethidium bromide. (**B**) Autoradiogram of the same gel. (**C**) The other half was fractionated on a 0.7% alkaline agarose gel to visualize leading and lagging strand synthesis by autoradiopgraphy. Leading strand products mostly centered around 3-kb, corresponding to the full size of the plasmid. The minor band detected at 6-kb may correspond to replication of a plasmid dimer that is minority present in our plasmid preparation. M: 3´-labeled λ DNA marker. LRI, Late replication intermediates; FI, FIII: Forms I and III of plasmid DNA, respectively.

Supplementary Figure S4

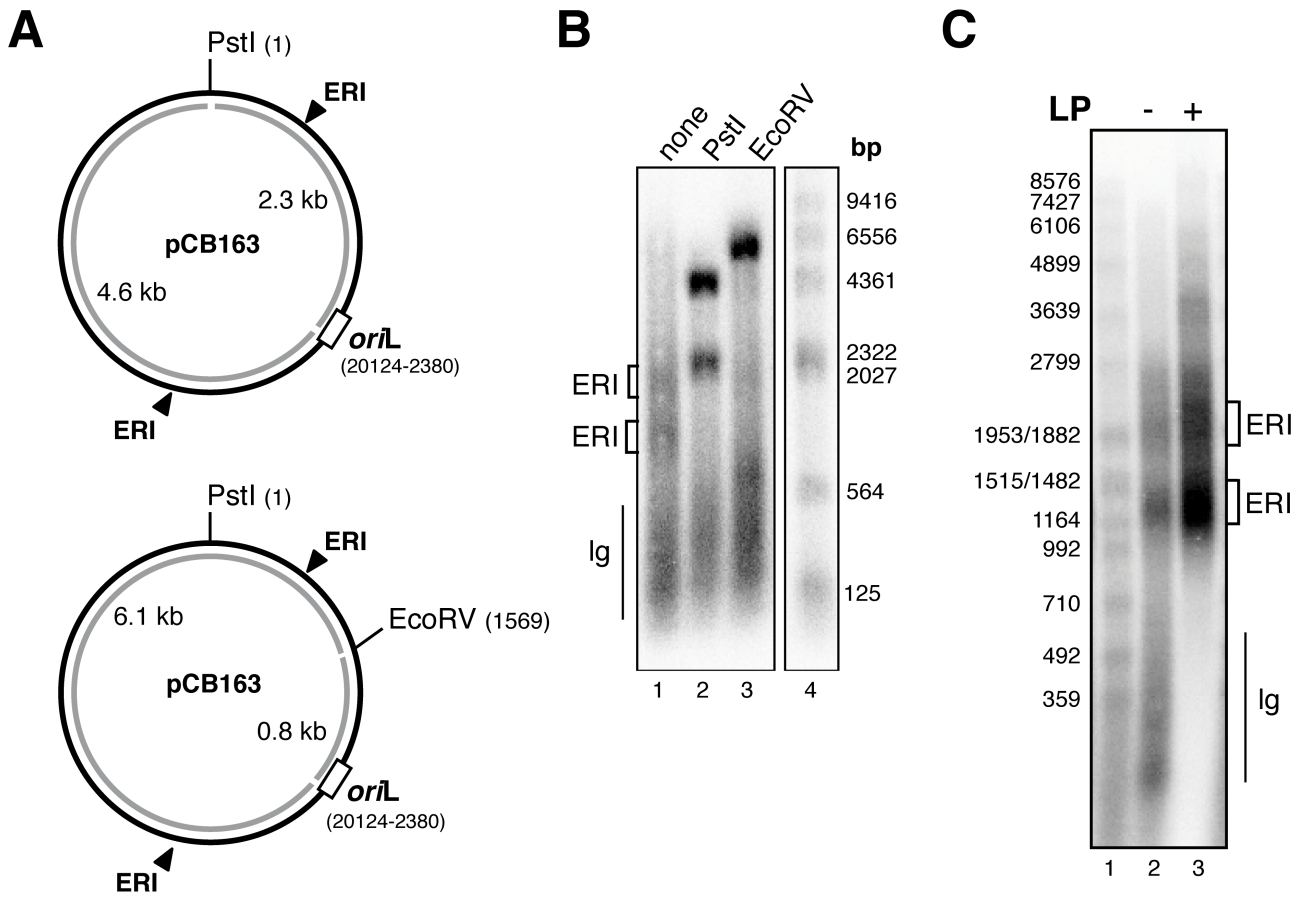

Figure S4. DNA replication starting from *ori*L is unidirectional and proceeds in some molecules clockwise and in others counterclockwise. (**A**) In the absence of gyrase, DNA replication starts but stalls due to the accumulation of positive supercoiling, and early replication intermediates (ERI) are formed. Scheme of the run-off leading strand replication products expected after removal of torsional stress by *Pst*I (top) or *Eco*RV (bottom) digestion. The putative localization of the ERIs is also shown (triangles). (**B**) Replication run-off reactions confirm that DNA synthesis starts from *ori*L in pCB163. Standard θ-type replications reactions were set up in the absence of DNA gyrase and were incubated for 4 min to allow replication start and ERIs formation. Then 1U of the indicated restriction enzyme was added to allow removal of torsional stress. Reactions continued for 15 min to run-off replication up to the restriction site, and the replication products were analyzed by denaturing alkaline gel electrophoresis and autoradiography. (**C**) DNA replication starting from *ori*L is unidirectional. Standard θ-type replications reactions in the absence of DNA gyrase were incubated for 4 min to allow DNA replication start and accumulation of ERIs. Then DNA Polymerase I and T4 DNA ligase were added. Reactions continued for another 15 min, to allow the completion and ligation of replication products synthesized on the same template. After deproteinization, products were analyzed by denaturing alkaline gel electrophoresis and autoradiography. Abbreviations: ERI: early replication intermediate, lg: lagging strand products, LP: Ligase and Pol I. See supplementary text for more details.

Supplementary Figure S5

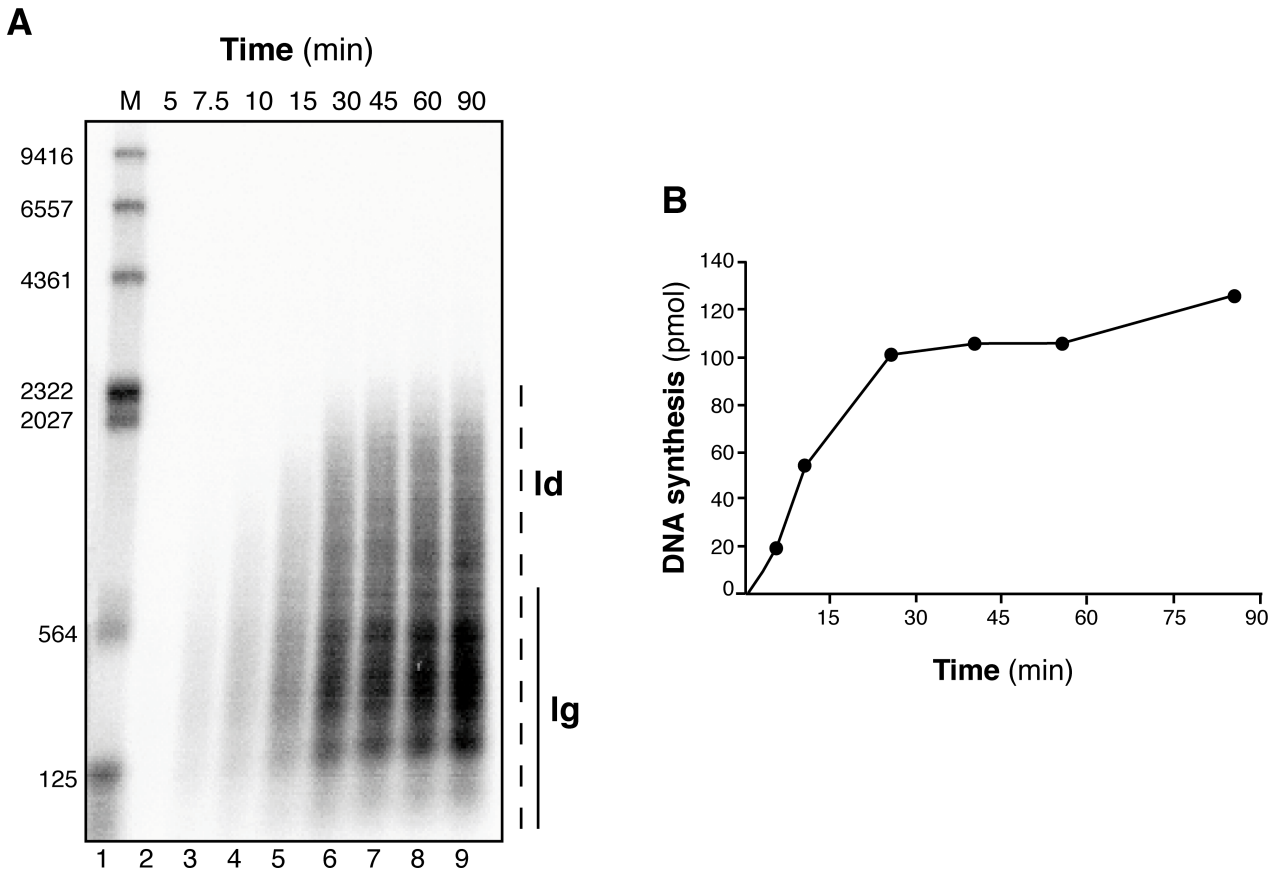

Figure S5. Progression over the time of *ori*L-dependent replication reactions performed with DnaE as the sole DNA polymerase. (**A**) Alkaline gel showing the replication products obtained over time in reactions with pCB163 as the DNA template. All replisome components except PolC were present. ld: leading strand, lg: lagging strand. M is *Hin*dIII labelled λ DNA. (**B**) The amount of DNA synthesized with DnaE as the sole DNA polymerase is plotted versus the time of incubation.

Supplementary Figure S6

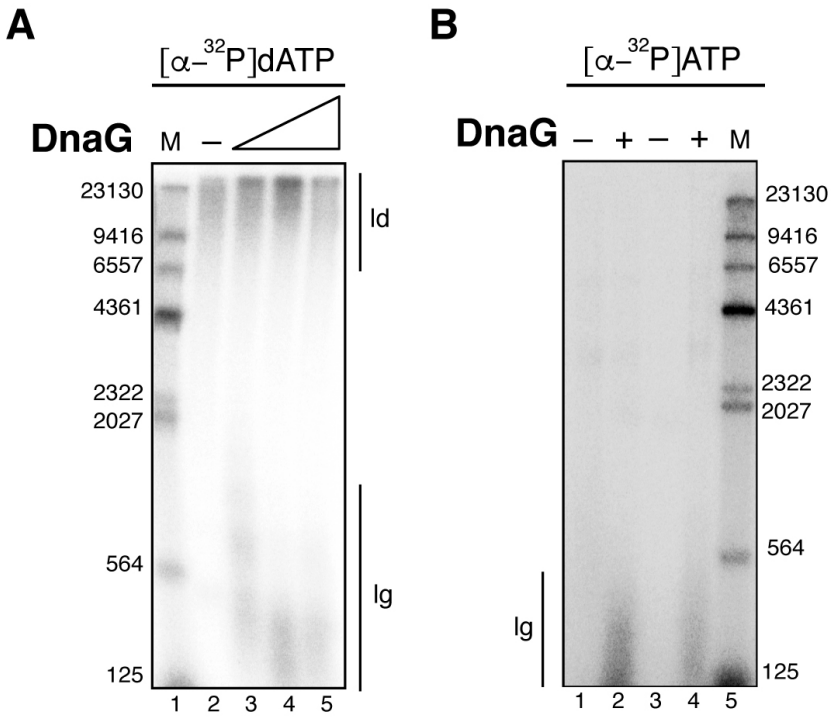

Figure S6. Effect of DnaG in σ-type replication reactions with PolC as the only polymerase. (**A**) Increasing concentrations of DnaG stimulate leading strand synthesis and decrease the size of the Okazaki fragments synthesized by PolC. Alkaline gel showing the leading and lagging replication products obtained in reactions preformed with the synthetic nicked mini-circle substrate and all replisome components except DnaE (i.e, PolC was as the sole DNA polymerase). DnaG primase concentrations varied from 20 to 80 nM. Reactions were incubated during 15 min at 37ºC and were done with [α-^{32}P]dATP as the radiolabelled nucleotide to visualize both, leading and lagging strand replication. (**B**) [In reactions where PolC is the sole DNA polymerase, [α-^{32}P]ATP ribonucleotide is only incorporated into the lagging strand. Reactions were performed with the synthetic nicked mini-circle template with [α-^{32}P]ATP as the only radiolabeled nucleotide, in a 1*:1000 [α-^{32}P]ATP:ATP ratio. All replisome components were present, except DnaE (i.e, 20 nM PolC was the only DNA polymerase), and when indicated 40 nM DnaG (+ symbol) was also added. The reaction was performed in duplicate. Following incubation for 15 min at 37 ºC, the samples were deproteinized and the products were analyzed by alkaline gel electrophoresis and autoradiography. Abbreviations: ld: leading strand product, lg: lagging strand products. M is *Hin*dIII labelled λ DNA.

Supplementary Figure S7

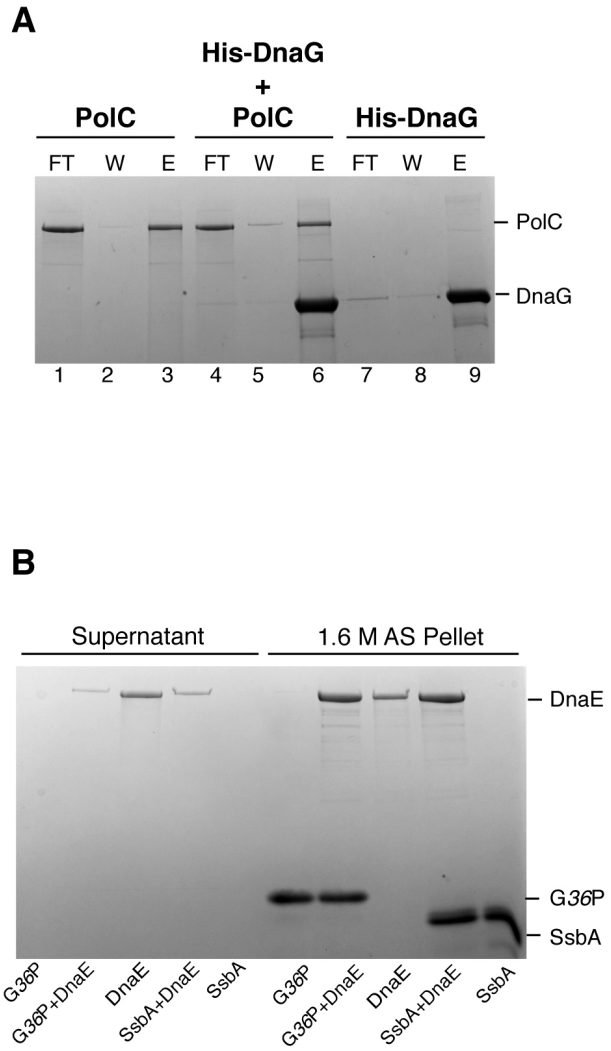

Figure S7. Protein-protein interactions at the SPP1 replisome. (**A**) PolC does not interact with DnaG. Purified N-terminal His-tagged DnaG and native PolC (3 µg each) were incubated in 100 µl of binding buffer, and then the protein mixtures (N-His-tagged DnaG, PolC, and N-His-tagged DnaG+PolC) were incubated with 20 µl of pre-equilibrated nickel-magnetic beads for 10 min at 4ºC with gentle mixing. After washing the beads, the bound proteins were eluted with 30 µl of SDS-PAGE sample loading buffer. Flowtrough (FT), wash (W), and elution (E) fractions were analyzed by SDS-PAGE and Coomassie blue staining as described in the supplementary methods. (**B**) DnaE interacts with SsbA and with G*36*P. Purified DnaE was incubated with G*36*P or SsbA (3 µg each) for 15 min at 4 ºC in 40 µl of binding buffer, and then 100 µl of precipitation buffer was added to the proteins mixtures to give an ammonium sulfate concentration of 1.6 M. After 10 min at 4ºC, samples were centrifuged for 5 min at 14000 rpm, and the pellets were washed once with 200 µl of precipitation buffer. The pellets were resuspended in 30 µl of SDS-PAGE sample loading buffer. A Coomasie blue stained gel is shown with the Supernatant (left) and Pellet (right) of the different protein mixtures. See supplementary methods for further explanations. Abbreviation, AS: ammonium sulfate.

Supplementary Figure S8

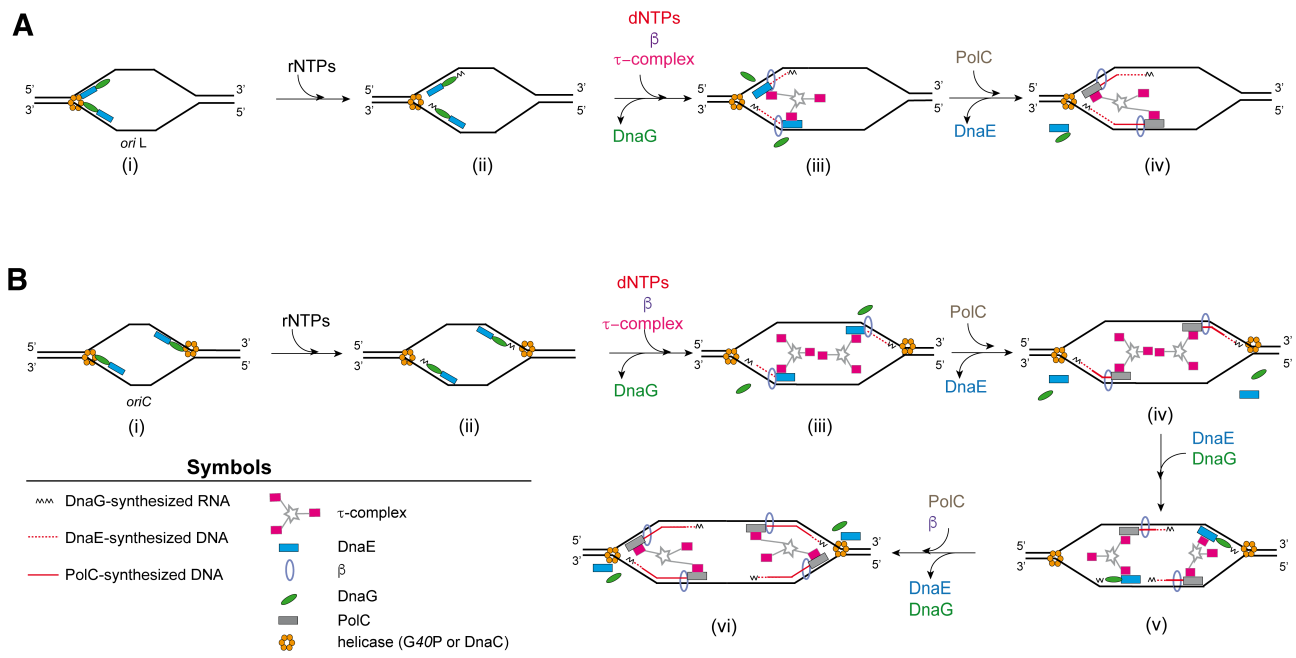

Figure S8. Model of initiation of θ-type DNA replication, showing the division of labour between the two replicative DNA polymerases present in Firmicutes. (**A**) Initiation of unidirectional DNA replication in phage SPP1. (i) The helicase G*40*P binds to the *ori*L region by the aid of the G*38*P and G*39*P. (i). G*40*P recruits the host DnaG primase and DnaE polymerase, forming the primosome. Two DnaG-DnaE complexes are loaded, complexed with one replicative helicase. (ii) In the presence of rNTPs, a RNA-primer is formed at both strands. (iii) In the presence of dNTPs, and aided by the β clamp and the τ-complex, DnaE extends the RNA primer forming an RNA-DNA hybrid on both strands. DnaG might leave the complex. (iv) The two RNA-DNA hybrids are rapidly handed off to PolC, which continues both leading and lagging strand synthesis. We postulate that the viral SSB (G*36*P) and DnaG contribute to the mechanism of primer handoff. DnaG and DnaE may remain complexed with the helicase or re-loaded just on the lagging strand for the next Okazaki synthesis. (**B**) A model showing the initiation of bidirectional DNA replication in Firmicutes. At the bacterial origin of replication (*oriC*), two helicases (DnaC) are loaded by the host helicase loaders (DnaA, DnaB, DnaD, DnaI). (i) Bidirectional θ-type DNA replication can then start by DnaC-mediated loading of two DnaE-DnaG complexes at each site, and bidirectional replication occurs as in (A) but with two replication forks. Alternatively, as it is depicted here, only one DnaE-DnaG complex is loaded at each site, forming two primosomes. (ii) In the presence of rNTPs, a RNA-primer is formed at both strands. (iii) In the presence of dNTPs, and aided by the β and τ-complex, DnaE extends both RNA primers forming RNA-DNA hybrids. DnaG might leave the complex. (iv) The RNA-DNA hybrids are rapidly handed off to PolC, and DnaE might leave the fork. Then, the loaded PolC polymerase becomes the leading strand polymerase of the opposite moving fork. (v) At each fork DnaE and DnaG are again loaded to prime lagging strand replication. (vi) At the lagging strand, PolC displaces the DnaE polymerase and synthesizes also the lagging strand. An additional β-clamp might be loaded at this step to reconstitute finally two complete replisomes moving in opposite directions. For simplicity the replisome organizers (G*38*P and DnaA), the helicase loaders (G*39*P and DnaD-DnaB-DnaI) and the SSBs (G*36*P and SsbA) are not shown in the figure.