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Involvement of DnaE, the Second Replicative DNA Polymerase from *Bacillus subtilis*, in DNA Mutagenesis*

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In a large group of organisms including low G + C bacteria and eukaryotic cells, DNA synthesis at the replication fork strictly requires two distinct replicative DNA polymerases. These are designated pol C and DnaE in *Bacillus subtilis*. We recently proposed that DnaE might be preferentially involved in lagging strand synthesis, whereas pol C would mainly carry out leading strand synthesis. The biochemical analysis of DnaE reported here is consistent with its postulated function, as it is a highly potent enzyme, replicating as fast as 240 nucleotides/s, and stalling for more than 30 s when encountering annealed 5′-DNA end. DnaE is devoid of 3′ → 5′-proofreading exonuclease activity and has a low processivity (1–75 nucleotides), suggesting that it requires additional factors to fulfill its role in replication. Interestingly, we found that (i) DnaE is SOS-inducible; (ii) variation in DnaE or pol C concentration has no effect on spontaneous mutagenesis; (iii) depletion of pol C or DnaE prevents UV-induced mutagenesis; and (iv) purified DnaE has a rather relaxed active site as it can bypass lesions that generally block other replicative polymerases. These results suggest that DnaE and possibly pol C have a function in DNA repair/mutagenesis, in addition to their role in DNA replication.

In all living organisms, DNA replication is carried out by a functionally highly conserved protein complex. Genetic and biochemical data have shown that this complex, called DNA polymerase holoenzyme, contains two copies of an essential replicative DNA polymerase in *Escherichia coli*, T4 and T7 phages, and SV40 (reviewed in Refs. 1–6). In contrast, replication requires two different polymerases in bacteria *Bacillus subtilis* and *Staphylococcus aureus* (pol[1] C and DnaE, C family (7, 8)) and in eukaryotes including *Saccharomyces cerevisiae*, *Xenopus*, and human (pol δ and pol ϵ, B family; reviewed in Refs. 5 and 9–11). Thus, holoenzyme of these organisms might be more complex, containing two different polymerases instead of two copies of a single polymerase. This higher level of complexity would hold true for many organisms as follows: (i) systematic sequencing of bacterial genomes (more than 100 completed to date) revealed that ~50% carry at least two copies of *dnaE* or contain *dnaE* and *polC* (no genome containing only *polC* has been detected so far), and (ii) pol δ and pol ϵ seem to be ubiquitous in eukaryotes.

It is well established that pol C in bacteria and pol δ in eukaryotes are required at the replication fork (5, 9–15). On the other hand, the specific roles of DnaE and pol ϵ during replication are still not known. In *B. subtilis*, it was reported that the purified DnaE protein has a DNA polymerase activity devoid of proofreading activity and presents a high affinity for dNTP (14, 16). Genetic and cytological data as well as *in vivo* assays of radioactive precursor incorporation have shown that DnaE, like pol C, is essential for the elongation phase of replication and is associated with the replication factory at mid-cell (7). Moreover, study of plasmid replication intermediates indicated that DnaE plays a role in lagging strand synthesis, whereas pol C is mainly involved in leading strand synthesis (7). These results suggest that two different replicative polymerases ensure synthesis of both DNA strands in *B. subtilis* and, by extension, potentially in all organisms encoding these two polymerases. Interestingly, genes are mostly (78%) colinear with the replication forks in bacteria encoding both pol C and DnaE, whereas they are much more randomly oriented in organisms carrying only the *dnaE* gene (17), suggesting a different organization of the replication fork in these bacteria.

In this work, we show that *B. subtilis* DnaE is an efficient DNA polymerase, with moderate processivity and no 3′ → 5′-exonuclease activity. By using templates bearing miscoding or non-coding lesions, we have observed that DnaE is able to bypass AAF adducts and abasic sites (although less efficiently) but not (6-4)TT photoproducts and BaP adducts and that it produces mainly frameshifts rather than base substitution. Furthermore, *dnaE* transcription and translation are induced ~3-fold during the SOS response. Interestingly, UV-induced mutagenesis is abolished upon DnaE depletion. These results suggest that DnaE could have dual role in *B. subtilis*, one with pol C in chromosome replication and the other in DNA mutagenesis during the SOS response.

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[1] The abbreviations used are: pol, DNA polymerase; Klenow, the Klenow fragment of pol I; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; nt, nucleotide(s); BaP, benzo-(*a*)-pyrene; AAF, *N*-acetylaminofluorene; AP, abasic site; (6-4)TT photoproduct, thymine-thymine pyrimidine(6-4)pyrimidone photoproduct; TLS, translesion synthesis; IPTG, isopropyl-β-D-thiogalactoside; DTT, dithiothreitol; BSA, bovine serum albumin; WT, wild type.

EXPERIMENTAL PROCEDURES

Strains and Expression Vector—B. subtilis strains are all derivatives of strain 168. In HVS614, the *dnaE* gene is placed under the control of the P*spac* promoter (7). This strain was obtained by single crossover integration of pMUTIN2 carrying a DNA segment overlapping the 5′-end of the *dnaE* gene and harbors pMAP65, a plasmid overproducing the LacI repressor (18). This strain thus allows (i) the tight regulation of the P*spac* activity upon addition of isopropyl-β-D-thiogalactoside (IPTG) and (ii) the measure of *dnaE* expression using *dnaE*:*lacZ* transcriptional fusion. HVS609 carries a similar P*spac-pol C* fusion (7). The *dinR*-deficient (DinR$^-$) strain, carrying the interruption mutant *dinR*::*upp-K7*, was previously published (19). *E. coli* B834(DE3) (*hsdS gall cIts857 ind1 metSam7 nin5 lacUV5-T7 gene 1* (20)) was used to overexpress DnaE.

DnaE protein was purified using an expression vector pTYB3*dna*E derived from the pTYB3 vector (IMPACT-CN system from New England Biolabs). It allows the expression of a C-terminal fusion protein between DnaE and a self-cleavable intein/chitin binding domain tag. Expression of the fusion protein is under the control of the $P_{T7/lac}$ promoter. The *dnaE* open reading frame was amplified by PCR from the strain *B. subtilis* 168 using the following primers: P100, 5′-aaggaaaaacATG TCT TTT GTT CAC CTG CAA GTG (containing a BspLu11I restriction site, underlined, compatible with the NcoI site; the sequence of the *dnaE* gene is in capital letters); and P101, 5′-ggtggttgctcttcgcaCCA CTG TTT TAA AAC GAC GTT TTT TTG (containing a SapI site). The PCR product was digested by BspLu11I and SapI and cloned into the NcoI and SapI sites of pTYB3. Oligonucleotides were from Genset or Genosys.

Proteins—pol I Klenow fragment and T7 pol were purchased from Roche Applied Science and New England Biolabs, respectively. DNA sequencing was carried out with Sequenase™ version 2 (APBiotech) according to the manufacturer. For all polymerases, 1 unit of enzyme catalyzes the incorporation of 10 nmol of total nucleotide into acid-insoluble material in 30 min at 37 °C. *E. coli* SSB was purchased from U. S. Biochemical Corp. Proteinase K was from Roche Applied Science. *B. subtilis* SSB was a gift from P. Polard.

Chemicals—[α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were purchased from PerkinElmer Life Sciences. Unlabeled nucleotides were from Amersham Biosciences. Heparin sodium was from Roussel UCLAF.

Purification of the B. subtilis DnaE Protein—B834(DE3) cells freshly transformed with pTYB3*dnaE* were grown to mid log phase at 30 °C in LB medium supplemented with 100 μg/ml ampicillin. At A_{650} = 0.6, expression was induced by addition of 1 mM IPTG for 4 h. It allows the overproduction of the *dnaE-Intein-CBD* (where CBD is chitin binding domain) fusion protein to about 1% of total protein in a soluble form. All the following operations were carried out at 4 °C. The cell pellet was resuspended in 50 ml of buffer TEN_{1000} (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 M NaCl), sonicated, and centrifuged at high speed to yield a clear lysate. The clear lysate was subsequently loaded in batch onto 8-ml chitin beads (New England Biolabs). Beads were washed 3 times with 100 ml of buffer TEN_{2000} (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 M NaCl) to get rid of unbound proteins. Intein-mediated self-cleavage was performed by an on-column incubation of the immobilized fusion protein for 42 h with 30 mM DTT in buffer TEN_{1000}. This resulted in the release of 80% pure wild-type DnaE protein. The protein was then further purified by chromatography. DnaE was dialyzed overnight against buffer TEN_{50} supplemented with 10% glycerol, loaded onto a Hi-Trap Q column (Amersham Biosciences) to eliminate the contaminant DNA, and eluted with a linear NaCl gradient (DnaE elutes at about 215 mM NaCl). The fractions containing DnaE were dialyzed overnight against buffer TEN_{50} supplemented with 10% glycerol, loaded onto a Hi-Trap heparin column (Amersham Biosciences) to eliminate the main contaminant proteins, and eluted with a linear NaCl gradient (DnaE elutes at about 235 mM NaCl). Salt concentration was increased up to 300 mM NaCl to prevent DnaE precipitation. The protein was then stored at −80 °C in buffer TEN_{300} supplemented with 10% glycerol and 5 mM DTT. The yield of DnaE was about 4 mg of protein per liter of culture, and its purity was estimated to be 95% after SDS-PAGE and SYPRO Red (Molecular Probes) staining. Protein concentrations were quantified using the Coomassie Protein Assay Kit (Pierce) using BSA as a standard. A gel filtration chromatography was performed using a Superose 12 column (Amersham Biosciences) and buffer TEN_{50} supplemented with 10% glycerol, to determine the oligomerization state of DnaE. DnaE eluted as a dimer on the gel filtration column.

Primer Extension Reactions—A ^{32}P-labeled 30-mer primer P109, 5′-gtaccccggttgataatcagaaaagcccca, was annealed to M13mp18 ssDNA templates (New England Biolabs). Kinetics of primer extension were performed in 60 μl containing 75 ng of primed ssDNA and 60 μM of each dNTP. SSB was added to the reaction mixture as indicated in the figure legends. Reactions were preincubated 5 min at 30 °C in the presence or in the absence of SSB, with all the other components, before DNA polymerase addition. Reaction buffer contained 20 mM Tris-HCl, pH 7.5, 12.5 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 25 μg/ml BSA, and 2% glycerol. At the time indicated in the figures, 10-μl aliquots were taken, and DNA synthesis was stopped by the addition of 25 mM EDTA and 500 μg/ml proteinase K. The mixture was then incubated for 15 min at 55 °C. Reaction products were analyzed by electrophoresis on 0.8% agarose gels (Seakem GTG) under native conditions in TAE buffer (40 mM Tris acetate, pH 8.3, 1 mM EDTA) at 2 V/cm for 16 h. DNA was visualized and analyzed on a STORM apparatus (Amersham Biosciences).

Exonuclease Activity—Reactions of 10 μl contained 20 fmol of ^{32}P-labeled primer P109 in 20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 25 μg/ml BSA, 1% glycerol, and either 0.5 units of Klenow or 40 fmol of DnaE. At the time indicated in the figure legends, the samples were processed as described above and analyzed on a 12% denaturing (8 M urea) polyacrylamide gel. Electrophoresis was carried out at 60 watts/100 mA for 3 h in TBE buffer (90 mM Tris borate, pH 8.3, 2 mM EDTA).

Measurement of Strand Displacement Activity—The procedure used to measure the strand displacement activity has been described previously (21). Briefly, a ^{32}P-radiolabeled primer 1212 was annealed to a ssM13mp18 at positions 6326–6310 in 2-fold molar excess. In addition, one of the two following unlabeled oligonucleotides was annealed to ssM13mp18 at positions 6235–6216 in a 2-fold molar excess. Primer 45 (5′-GAA TTC GTA ATC ATG GTC AT-3′) is complementary to the template. Primer 37 (5′-CTA ATC AGG AGA ATT CGT AAT CAT GGT CAT-3′) is identical to number 45 for its 3′-end but possesses at its 5′-end an extra heterology of 10 bases. T7 pol, known to be devoid of strand displacement activity in the absence of SSB (21), was used to test the quality of the substrates. Primer extension reactions were performed as described above, except that they contained 125 ng of doubly primed M13mp18 ssDNA in 50 μl. Aliquots of 5 μl were withdrawn at the indicated times and processed as described above.

Enzymatic Assays—β-Galactosidase was assayed as described previously (22). β-Galactosidase activities are expressed in Miller units per mg of protein. Protein concentrations were determined using the Coomassie Protein Assay Kit (Pierce) using BSA as a standard.

Immunodetection of $DnaE_{Bs}$—Immunization against $DnaE_{Bs}$ and serum preparation in the rabbit was entrusted to Eurogentec. Prior to injection, $DnaE_{Bs}$ protein was further purified to homogeneity by SDS-PAGE. DnaE levels in the different strains and growth conditions were determined by immunoblot analysis essentially as described previously (23) except for the following modifications. Equal amounts of soluble cellular proteins (equivalent to 3.7×10^7 cells) were separated by 8% SDS-PAGE and transferred to a Hybond PVDF membrane (Amersham Biosciences) by electroblotting using HorizBlot semi-dry transfer system (Atto Instruments). DnaE antibodies were diluted 1:500. Protein G-horseradish peroxidase (Bio-Rad, 1:5000 dilution) was used as a secondary antibody. DnaE was revealed using the ECL+ reagent (Amersham Biosciences) and a Storm apparatus (Amersham Biosciences) and quantified using ImageQuant software.

Measure of Spontaneous and UV-induced Mutagenesis—Overnight cultures grown in LB (168) or in LB supplemented with erythromycin (0.6 μg/ml), kanamycin (5 μg/ml), and IPTG (25 μM for *spac-DnaE* or 100 μM for *spac-pol C*) were diluted 1000-fold in fresh media supplemented or not with IPTG at the concentration indicated in Fig. 6*A*. At an A_{650} of 0.3–0.4, cells from 10-ml aliquots were pelleted by centrifugation and resuspended in 26 ml of minimal salts (supplemented when necessary with IPTG at the same concentration as in the culture). Half of the cells were irradiated at 80 J/m^2 in a Petri dish upon gentle agitation. Both irradiated and unirradiated cells were pelleted by centrifugation, resuspended in 5 ml of fresh LB media similarly supplemented with antibiotics and IPTG when required, and incubated at 37 °C until cultures enter stationary phase.

UV sensitivity was measured immediately after UV irradiation, plating serial dilution of the cultures on LB agar plates supplemented as in the liquid cultures. Mutagenesis was estimated by determining the frequency of rifampicin-resistant cells (at 10 μg/ml rifampicin) present in unirradiated or irradiated samples at the end of the culture.

TLS Substrates—Construction of single-stranded plasmids containing a lesion at a specific position (pUC-L) has been extensively described previously (24, 25). Lesions used in this study and their se-

quence context were described previously: AAF adducts (26–28), (6-4)TT photoproduct (29), and BaP adduct (30). The presence of the lesion on all the template molecules was verified during the construction process by restriction as described previously (24, 25). For the AP site-containing template, 90-mer oligonucleotides containing a single AP site within the NarI sequence context (5′-GGC*X*CC-3′, where *X* represents the abasic site) were purchased from Eurogentec.

Lesion Bypass Assays—For all the TLS experiments except AP site-containing templates, assays were performed using single-stranded substrates pUC-L primed with the oligonucleotide 2316, 5′-ACAC-GACGTTCCGCTAATTCAACC-3′, that hybridizes 90 bases upstream from the lesion. For the AP site-containing template, the primer used was NarL-1, 5′-TGCCAAGCTTAGTCTGTGG-3′, that anneals 1 base before the lesion on the 90-mer template. All reactions were conducted at 37 °C in 20 μl of the following buffer: 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.1 mg/ml BSA, and 100 μM of each dNTP. SSB (1 molecule of SSB as a monomer/nt, oversaturating amount) was added when using pUC-L substrate to prevent unspecific binding of the polymerase. Replication bypass was performed by incubating 1 fmol of primer/template (pUC-L/2316* or 90-AP/NarL-1*) with 1, 5, and 10 fmol of DnaE for 10 min at 37 °C. Reactions were stopped by addition of 20 mM EDTA and 4 volumes of termination mixture (4 μg of proteinase K, 1% SDS), incubated for 1 h at 37 °C, and ethanol-precipitated. The pellet was then redissolved and restricted for 3 h by EcoRI to determine the length of the bypass products. EcoRI restriction cleaves 11–13 nucleotides downstream from the lesion on the pUC-L templates, thus generating shorter fragments that can be separated by PAGE. No restriction was performed on the 90-mer AP templates. Restriction reactions were further ethanol-precipitated and resuspended in loading buffer (95% formamide, 5% TE, pH 8.0, 0.5% bromphenol blue). The reaction mixtures were heat-denatured, electrophoresed for 3–4 h (2000 V/80 watts) in TBE buffer on 12 or 15% denaturing (8 M urea) polyacrylamide gels, and visualized using a PhosphorImager 445SI (Amersham Biosciences).

Specificity of Deoxynucleotide Incorporation by DnaE Protein—^{32}P-Radiolabeled primers that annealed 1 base prior the lesion were used. DnaE (2.5 fmol) was incubated with the primer/template substrates (1 fmol) in the primer extension buffer as described above in the absence or in the presence of only one of each dNTP (G, A, T, or C) at 100 μM final concentration and incubated for 10 min at 37 °C. Reactions were stopped by adding 20 mM EDTA and 4 volumes of loading buffer, electrophoresed on 20% denaturing (8 M urea) polyacrylamide gel, and visualized as described above.

RESULTS

DnaE Is a Potent DNA Polymerase Lacking 3′ → 5′-Exonuclease Activity—To explore the biological function of *B. subtilis* DnaE, the enzyme was purified to homogeneity from *E. coli* (see "Experimental Procedures"). DNA synthesis on a primed ssDNA showed that DnaE fully replicates the 7.25-kb M13mp18 genome at a speed of 60 nt/s at 37 °C (Fig. 1*A*, *left*). Coating of the template with SSB from either *E. coli* or *B. subtilis* enhances the rate of synthesis to 120 nt/s (Fig. 1*A*, *middle* and *right*). The rate of synthesis on *E. coli* SSB-coated ssDNA increases with the temperature from 60 nt/s at 30 °C to 240 nt/s at 47 °C (Fig. 1*B*). DnaE uses DNA or RNA primers with the same efficiency and is inhibited by NaCl concentration higher than 25 mM (not shown).

Protein sequence analysis suggests that *B. subtilis* DnaE does not contain the 3′ → 5′-exonuclease domain. This was experimentally confirmed as no DnaE-mediated degradation of a 5′-^{32}P-labeled oligonucleotide was observed (Fig. 1*C*). A similar result was obtained with an oligonucleotide annealed to an ssDNA template (not shown).

DnaE Is Poorly Processive—The processivity of DnaE was estimated by single-hit elongation experiments using heparin as a DNA polymerase trap. Preliminary experiments showed that DnaE is not a highly processive enzyme. Indeed, whereas T7 DNA polymerase (T7 pol) incubated with 2 μg of heparin fully replicates a primed M13mp18 ssDNA, 100 ng of heparin fully inhibited DNA synthesis with similar amounts of DnaE (data not shown). To determine more precisely the processivity of DnaE, time course experiments were performed, and extension products were analyzed by denaturing PAGE (Fig. 2). T7 pol and Klenow enzyme were used as controls. The lowest concentrations of enzymes required to efficiently convert a primed M13mp18 ssDNA into dsDNA were used (Fig. 2, *lanes 1* of each panel). The polymerases were incubated in the reaction mixture lacking $MgCl_2$, in order to allow DNA polymerase loading without polymerization. Simultaneously, 10 mM $MgCl_2$ and 800 ng of heparin were added to start the synthesis and trap the free and dissociated DNA polymerases, preventing them from (re)loading onto DNA. The efficiency of the trap was independently verified by two means. First, time course exper-

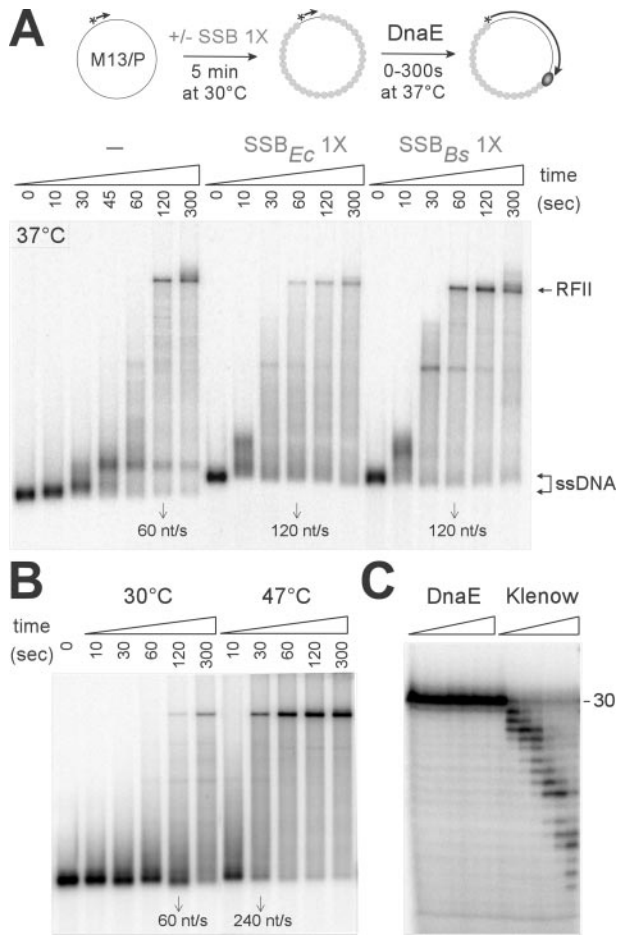

FIG. 1. **DnaE is an efficient DNA polymerase that lacks 3′ → 5′-proofreading activity.** *A*, SSB effect on the rate of synthesis of DnaE. *Top*, schematic representation of the experimental system. *Bottom*, kinetics of primer extension. Reactions were performed using an end-labeled oligonucleotide-annealed M13mp18 ssDNA either naked (*left*) or coated with saturating amounts of *E. coli* SSB (*middle*; 0.06 molecule of monomer of SSB_{Ec} per nt of template) or *B. subtilis* SSB (*right*, 0.1 molecule of monomer of SSB_{Bs}/nt). The primed template was preincubated for 5 min at 30 °C with or without SSB in the reaction mixture and then transferred at 37 °C before DnaE addition. Aliquots were removed at the indicated times (10–300 s), and the products were separated by electrophoresis on native agarose gels. Each lane contains 5 fmol of template and 290 fmol of DnaE. Positions of the naked or SSB-coated labeled template (ssDNA) and the fully replicated product (RFII) are indicated. *B*, temperature effect on the rate of synthesis of DnaE. Kinetics of primer extension were performed at either 30 or 47 °C (note that a rate of synthesis of 240 nt/s was obtained for temperatures ranging from 42 to 62 °C). Reactions were as described in *A* using a primed M13mp18 ssDNA coated with saturating amount of *E. coli* SSB as template. Each lane contains 5 fmol of template and 37 fmol of DnaE. *C*, analysis of the 3′ → 5′-exonuclease activity of DnaE and Klenow fragment. DnaE (40 fmol) or Klenow (0.5 units) were incubated with 20 fmol of 5′-^{32}P-radiolabeled 30-mer oligonucleotide at 37 °C. Aliquots were removed at the indicated time and analyzed on denaturing PAGE. Position of the 30-mer oligonucleotide is indicated on the *right*.

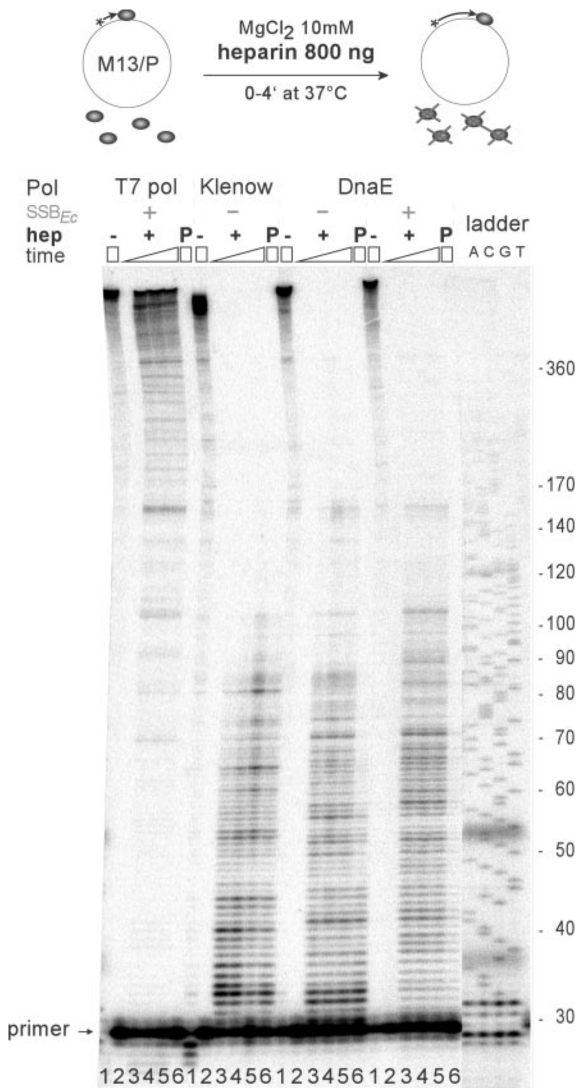

FIG. 2. **DnaE is a poorly processive DNA polymerase.** *Top*, schematic representation of the experimental system. Polymerase processivity was assessed using an end-labeled oligonucleotide annealed to M13mp18 ssDNA either naked or coated with saturating amounts of *E. coli* SSB. After preincubation of the primed template with or without SSB, an excess of polymerase was added and further incubated 3 min at 37 °C in a complete reaction mixture lacking $MgCl_2$. DNA synthesis was started by the simultaneous addition of $MgCl_2$ and 800 ng of heparin as a trap. Aliquots were withdrawn at different intervals, and replication products were analyzed by denaturing PAGE. The *diagonal lines* on the polymerases (*ovals*) indicate the trap action of heparin. *Bottom*, primer extension reactions. Either DnaE (100 fmol), T7 pol (100 fmol), or Klenow (300 fmol) were assayed on 4 fmol of DNA template. "SSB_{Ec}" indicates the absence (−) or presence (+) of saturating amounts of the *E. coli* SSB. *hep* indicates the absence (−) or presence (+) of heparin in the reaction mixture, or whether heparin was preincubated (*P*) with the polymerases before the addition of $MgCl_2$ to verify the efficiency of the trap. *Lane 1,* extension in the absence of heparin; *lanes 2–5*, 0-, 15-, 60-, and 240-s extension in the presence of heparin; *lane 6,* 240-s extension following preincubation of heparin with the polymerase. A sequencing reaction of M13mp18 with the universal −40 primer serves as a ladder. Position of the primer is indicated to the *left.*

iments were done to verify that the extension pattern did not change between 15 s and 4 min of incubation. Second, heparin was preincubated in the reaction mixture before addition of the polymerase to verify that it fully abolished DNA synthesis (Fig. 2, *panel T7 pol*, *lane 6*). In similar conditions, the processivity of Klenow was estimated to be 1–55 nt (Fig. *2*, *panel Klenow*, *lanes 3–5*), in full agreement with previous measures (31). For an unknown reason and as reported previously (32), heparin is not a perfect trap for Klenow polymerase (some synthesis is observed despite the heparin preincubation, Fig. 2, *panel Klenow*, *lane 6*). Nevertheless it can be used successfully to measure the processivity of the enzyme (extension profiles evolve little between 15 s and 4 min). The same procedure was applied to *B. subtilis* DnaE and revealed that the enzyme alone has a processivity similar to that of Klenow, the main signals indicating extension of 1–55 nt and faint signals up to 130 nt (Fig. 2, *panel DnaE*, *lanes 3–5*). Coating the substrate with *E. coli* SSB slightly increased its processivity as main signals indicate extension of 1–75 nt, and faint signals were still observed up to 130 nt (Fig. 2, *panel DnaE* + SSB_{Ec}, *lanes 3–5*). SSB from *B. subtilis* gave similar results (not shown).

DnaE Pauses When Encountering Annealed 5′-Ends—In bacteria, Okazaki fragments are synthesized on the lagging strand of the replication fork every second at 37 °C. The lagging strand polymerase binds to the primer, synthesizes about 1000 nt, precisely terminates when encountering the RNA primer of the former Okazaki fragment, and rapidly dissociates from DNA. Considering the possible role of the *B. subtilis* DnaE polymerase in lagging strand synthesis, we tested its ability to pause in front of various 5′-ends, using a circular ssDNA template carrying two primers 91 nt away, as described previously (see Fig. 3 *top*) (21). Pausing was assessed in the absence or in the presence of SSB (either from *E. coli* or *B. subtilis*) at different concentrations. Upon initiation of DNA synthesis both annealed primers are elongated, and a dsDNA molecule is generated. Only the upstream primer is radioactively labeled, and replication from this primer is monitored at various time points, using denaturing PAGE. With a fully annealed downstream primer (primer 45) we observed a strong block of DnaE progression (Fig. 3, *B* and *C*). In the absence of SSB, DnaE pauses ≥8 min, although in its presence it pauses ~30 s. After pausing, DnaE progresses slowly through the dsDNA region indicating that it has a weak strand displacement activity. Similar results were obtained with a fully annealed RNA oligonucleotide (data not shown). When the downstream primer has a 5′-flapping tail (primer 37), no pause was observed (Fig. 3, *D–G*). Rather, a ladder of products longer than 91 nt was observed. This strand displacement activity, very weak in the absence of SSB (about 10 nt displaced per min, Fig. 3*D*), is clearly stimulated with *E. coli* SSB at saturating or above saturating amounts (Fig. 3, *F* and *G*). In these conditions, about 10 nt were displaced per s. A similar stimulation was observed with *B. subtilis* SSB at or above saturating concentrations, albeit the stimulation was weaker (data not shown). Thus, this analysis revealed that DnaE-mediated synthesis is strongly arrested at fully annealed DNA or RNA 5′-ends and that the polymerase has some strand displacement activity at 5′-flapping ends that is significantly enhanced by SSB.

DnaE Expression Is SOS-inducible—*Mycobacterium tuberculosis* contains two polymerases of the C family designated DnaE1 and DnaE2, DnaE1 being more homologous to *E. coli* and *B. subtilis* DnaE (37% identity) than DnaE2 (28% identity). Although DnaE1 is essential, loss of *dnaE2* function does not affect growth kinetics, and DnaE2 was shown to be required for drug- and radiation-induced mutagenesis (33). Moreover, as for *E. coli* pol II, pol IV, and pol V, involved in DNA repair/induced-mutagenesis, DnaE2 is SOS-induced (33, 34). Thus, to explore whether the replicative *B. subtilis* DnaE polymerase might be SOS-inducible, we analyzed *dnaE* transcription during the SOS response. For this purpose, cells carrying a transcriptional *dnaE-lacZ* fusion were treated with DNA-damaging agents known to induce the SOS response. Upon addition of mitomycin C, β-galactosidase activity increased 2.2-fold after ≥60 min of drug exposure (Fig. 4*A*). With nalidixic acid, a 3.1-fold increase was observed after ≥100 min of treatment. A similar increase

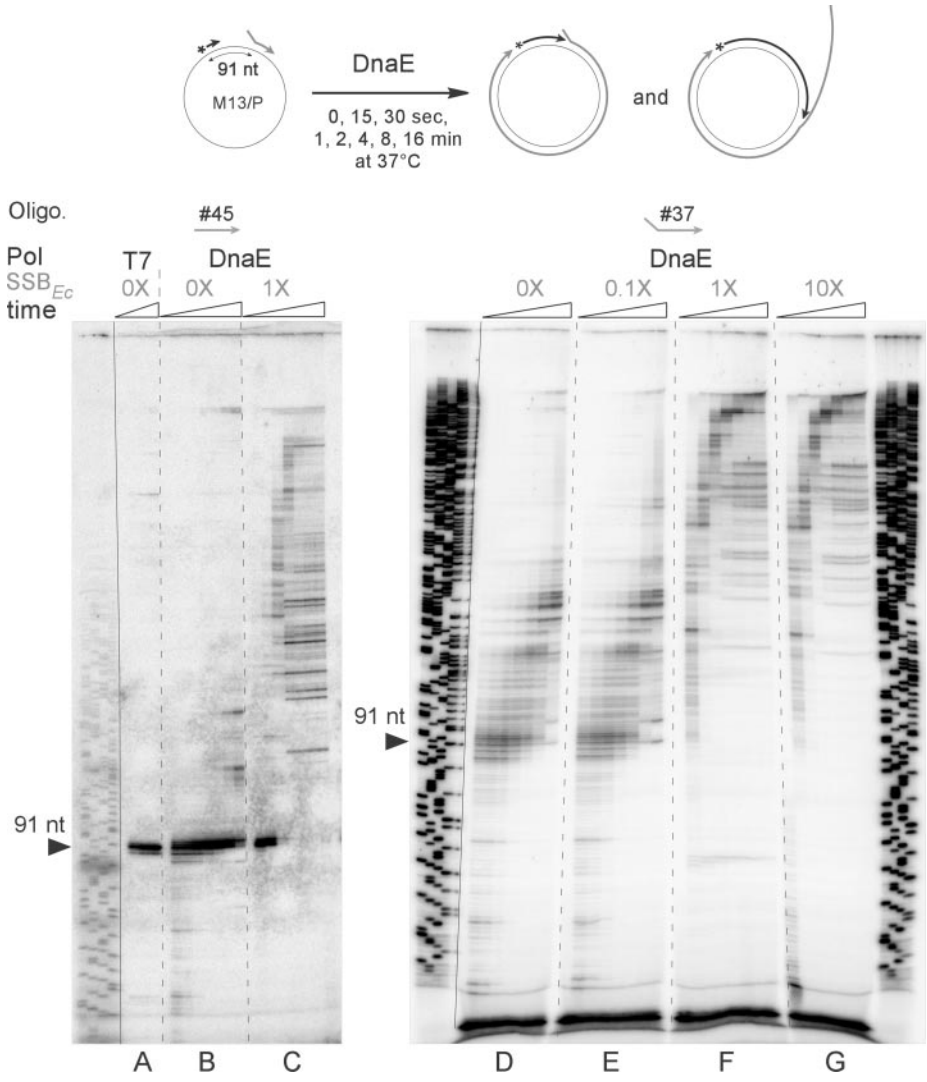

FIG. 3. **DnaE is transiently arrested when encountering 5′-ends.** *Top*, schematic representation of the experimental procedure. Primer extension reactions were performed as described in Fig. 1 except that the template carries 2 annealed primers. The labeled primer (number 1212) is indicated with an *asterisk*. The downstream primer is represented with a 5′-flapping tail. The *number within the circle* (*91 nt*) refers to the length of ssDNA that separates the two primers (including the 17 bases of the labeled primer). A polymerase devoid of any strand displacement activity is arrested upon encountering the annealed downstream primer, thus generating a labeled fragment of precisely 91 nucleotides. In contrast, a polymerase endowed with strand displacement activity progresses through the double-stranded region and generates labeled fragments of increasing length. Reaction times are indicated *below* the *straight arrow*. *Bottom*, primer extension reactions. Strand displacement activity was assayed at 37 °C using two alternative downstream primers: the 20-mer primer 45 fully complementary to the template (*panels A–C*) and the 30-mer primer 37, which carries an extra 10-base-long flapping tail at its 5′-end (*panels D–G*). Strand displacement activity was measured in the absence or in the presence of *E. coli* SSB, as indicated *above each panel*. The amounts of added SSB correspond to saturating amount (*1*×), 10 times less (*0.1X*), and 10 times more (*10X*). 56 fmol of DnaE and 5 fmol of template were used per reaction. T7 indicates a quality test of the template. It was performed using the T7 polymerase and primer 45 (45 fmol of T7 pol and 5 fmol of substrate were used per reaction). A similar result was obtained with primer 37, as published previously (21). A sequencing reaction of M13mp18 with the universal −40 primer was used as a ladder. An *arrow* indicates the transient block observed at 91 nt resulting from replication of ssDNA from the labeled primer 1212 up to the downstream primer.

was observed without any damaging agent in the *dinR* null isogenic strain (*dnaE-lacZ*, *dinR*$^-$; Fig. 4*A*), a strain inactivated for DinR, the functional analog of the *E. coli* LexA repressor that regulates expression of genes of the SOS regulon. To confirm these results, the amount of DnaE protein was measured by Western blot. In the wild-type 168 strain, a faint signal was observed, corresponding to ~300 molecules per cell (Fig. 4*B*). Treatment of this strain with mitomycin C or nalidixic acid led to a 6- and 3-fold increase in DnaE concentration, respectively. A 4-fold increase was observed in non-treated 168 *dinR*$^-$ cells. Altogether, these results show that DnaE belongs to the *B. subtilis* SOS regulon. Consistent with this conclusion, a putative DinR-binding site (GAACatttGTTT) was detected 74 bp upstream of the DnaE start codon.

Intracellular Concentration of DnaE Can Be Reduced About 4-fold Without Affecting DNA Synthesis—DnaE is estimated at ~300 copies in WT cells (see above). This value is rather high compared with the ~40 copies of the pol III core estimated in *E. coli* (35), but is only 3 times higher than the reported level of pol C (36). To determine the minimal amount of DnaE required for normal growth, a strain in which *dnaE* expression is under the control of the IPTG-regulated P*spac* promoter was used. In the absence of IPTG this strain stopped growing (Fig. 5*A*), formed filaments, and then died (not shown). At 10 μM IPTG cells were viable, as they could be propagated for at least 40 generations but might have somewhat deficient DNA synthesis, because they grew slower than WT cells, filamented weakly, and had a slightly reduced plating efficiency. At or above 25 μM IPTG growth was optimal, whereas at 15 μM IPTG filamentation and reduced plating efficiency were not observed, but the growth rate was not fully restored.

Western blot analysis showed that DnaE concentration varies with the IPTG concentration (Fig. 5*B*) from ~1100 copies per cell at 1 mM IPTG to ~350 copies per cell (a DnaE concentration similar to that of the WT strain) at 100 μM IPTG. Lowering IPTG concentration led to the progressive disappearance of the DnaE signal. To estimate DnaE concentration below 50 μM IPTG, we took advantage of the linearity of the P*spac* activity observed using similar fusions and IPTG concentration ranging between 5 and 200 μM (37). As expected, an ~2-fold reduction of DnaE was observed between 100 and 50 μM IPTG (Fig. 5*B*). We thus estimate that the minimal amount of DnaE required for optimal growth (at 25 μM IPTG) was ~80 and ~30 molecules for viability (at 10 μM IPTG). These results indicate that DnaE concentration can be reduced about 4-fold without affecting cell growth, suggesting that DNA replication is also unaffected.

Low Levels of DnaE or pol C Abolish UV-induced Mutagenesis—*E. coli* SOS-inducible polymerases (pol II, pol IV, and pol V) are involved in induced mutagenesis (38, 39). Additionally,

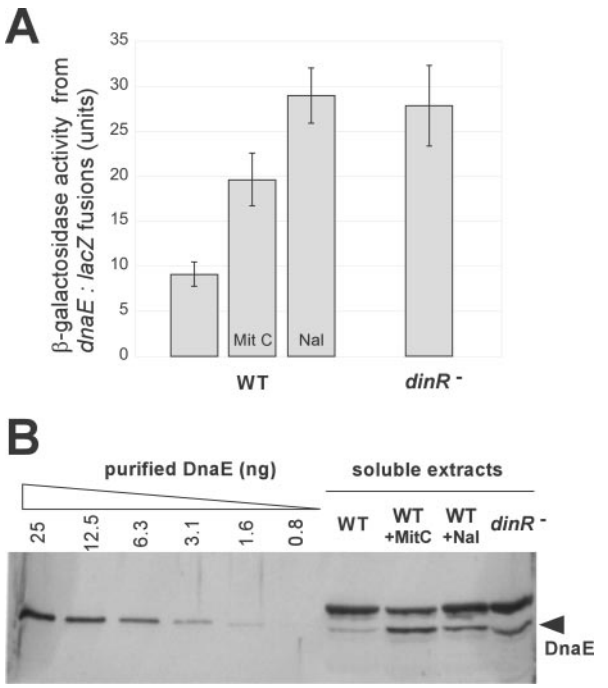

FIG. 4. **DnaE is a SOS-inducible polymerase.** *A*, β-galactosidase activity of *dnaE-lacZ* fusion strains. The effect of DNA-damage drugs or inactivation of the SOS repressor on *dnaE* expression was tested with *B. subtilis* cells carrying *dnaE-lacZ* fusions in a wild-type (*WT*) or *dinR*$^-$ background. Culture were grown in LB to A_{650} = 0.05–0.13 and then divided. The culture was either left untreated or exposed to DNA-damaging agents, mitomycin C (*Mit C*; 50 ng/ml) or nalidixic acid (*Nal*; 30 μg/ml). β-Galactosidase activity, monitored at different intervals after the drug addition, increases for 2 h and then reaches a plateau. The *diagram* and *vertical bar* represent the average and S.D. values obtained for at least 5 separate experiments, with samples recovered ≥2 h after drug addition. *B,* immunodetection of DnaE in *B. subtilis.* Cultures of either wild-type (*WT*) or *dinR*-deficient (*dinR*$^-$) *B. subtilis* cells were grown in LB to A_{650} = 0.15 and then divided. The cultures were either left untreated (WT, *dinR*$^-$) or exposed to DNA-damaging agents, as described in *A.* Cells were recovered at the end of the exponential phase (A_{650} = 0.5 for the untreated cells) or after 135 min of exposure to the drugs (A_{650} = 1.0 for *WT* + *Mit C*, A_{650} = 0.7 for *WT* + *Nal*). Cells were treated as described under "Experimental Procedures." Equal amounts of soluble cellular proteins (~3.7×10^7 cells for the WT untreated culture) were loaded in each lane. 0.8–25 ng of purified DnaE were loaded as a control. The *arrowhead* indicates the signal specific for the DnaE protein. The *upper band* results from the cross-reactivity of the DnaE antibodies against another *B. subtilis* protein present in the extracts. This cross-reactivity pre-existed in the preimmune serum. This band reproducibly decreased in intensity in the culture treated with mitomycin C.

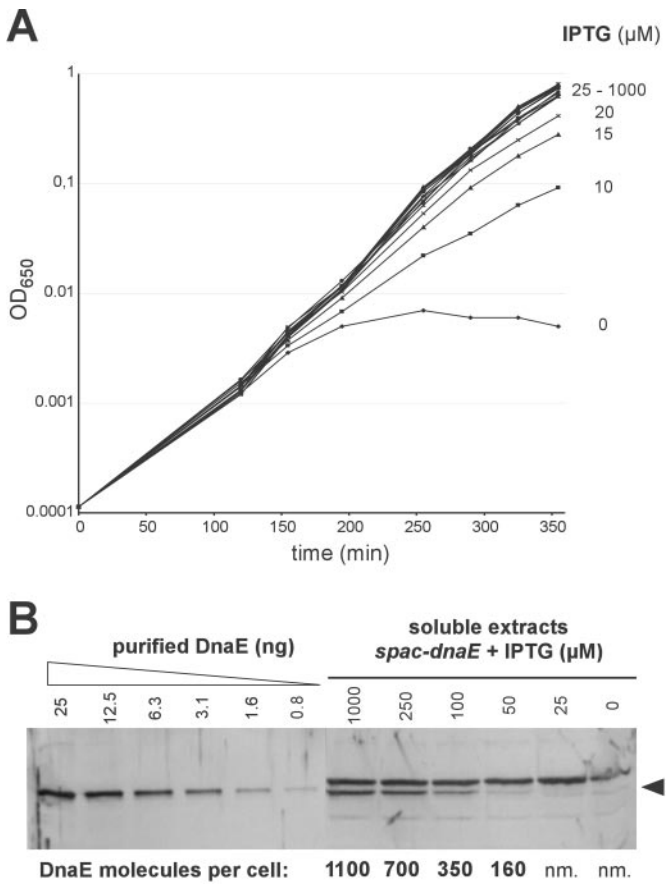

FIG. 5. **Determination of the minimal level of DnaE required for normal growth.** *A*, growth of *B. subtilis* cells carrying *spac-dnaE* fusion. Overnight HVS614 culture grown in LB supplemented with 25 μM IPTG were diluted periodically in fresh media supplemented with 0–1000 μM IPTG and optical density was followed for 350 min. *B*, immunodetection of DnaE in *spac-dnaE B. subtilis* cells. Overnight HVS614 cultures grown in LB supplemented with 25 μM IPTG were diluted 1000-fold in fresh media supplemented with 0–1000 μM IPTG. Cells were recovered at the end of the exponential phase (A_{650} = 0.4–0.6 after 225 min growth in the fresh media) and treated as described under "Experimental Procedures." Equal amounts of soluble cellular proteins (equivalent to ~3.7×10^7 cells for the cultures grown with IPTG) were loaded in each lane. *Numbers at the bottom* of the gel refer to the quantification of DnaE molecules per cell. *nm.*, non-measurable.

an error-prone activity was suggested for the SOS-inducible DnaE2 (C family) of *M. tuberculosis* (33). To test whether the SOS-inducible *B. subtilis* DnaE is involved in DNA repair/mutagenesis, we measured spontaneous and UV-induced mutagenesis in cells either depleted in (~30 copies/cell) or overexpressing (~1100 copies/cell) this protein. It is noteworthy that "overexpression" of DnaE corresponds to the DnaE amount present in WT cells upon SOS induction. Spontaneous mutagenesis (monitored as the proportion of Rif^R cells in the bacterial population, Fig. 6 *A*) and cell survival after UV irradiation at 80 J/m^2 (Fig. 6*B*) were not affected by variations in DnaE concentration. Remarkably, DnaE depletion prevented UV-induced mutagenesis (Fig. 6*A*). Indeed, whereas the proportion of Rif^R colonies increased ~20-fold upon irradiation in WT cells, the proportion remained at the basal level in irradiated depleted cells. As expected, high DnaE expression restored the UV-induced mutagenesis.

For comparison, a similar study was carried out with an isogenic strain encoding *polC* under the control of the IPTG-regulated P*spac* promoter. This strain was not viable at or below 30 μM IPTG. At 40 μM, cells were viable and could be propagated for at least 30 generations even though they had a slightly reduced growth rate and plating efficiency and filamented weakly (not shown). At 50 μM, growth rate and plating efficiently were restored to the level of WT cells, but a higher IPTG concentration (≥100 μM) was required to fully prevent filamentation. Together, these results indicate that pol C is significantly depleted at 40 μM IPTG and is present in sufficient amount at ≥100 μM. Interestingly, we found that variations in pol C concentration exhibit phenotypes similar to those observed with DnaE, as pol C depletion precluded UV-induced mutagenesis (Fig. 6*A*). The mean values of spontaneous and UV-induced mutagenesis are slightly higher in pol C-depleted cells than spontaneous mutagenesis in WT cells. This increase is however not statistically significant because standard deviations of the values overlap. Finally, it is noteworthy that the survival of pol C-depleted cells upon irradiation was about 5-fold lower than that of the wild-type or DnaE-depleted cells (Fig. 6*B*).

Bypass of DNA Adducts by DnaE—Results presented above show that UV-induced mutagenesis requires a relatively high

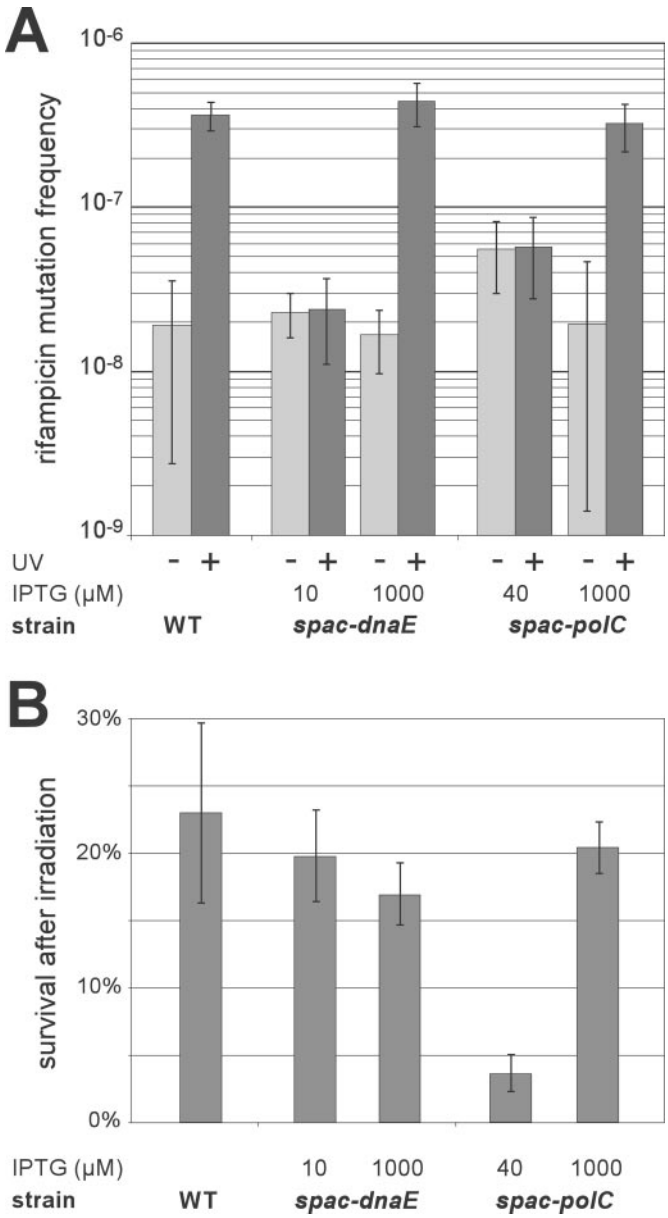

FIG. 6. **Mutagenesis and survival after UV irradiation of *B. subtilis* cells depleted or not for DnaE or pol C.** *A*, spontaneous and UV-induced mutagenesis. Average frequencies of rifampicin-resistant cells present in cultures submitted or not to UV irradiation at 80 J/m2. *Error bars* represent S.D. calculated using the data of at least six independent experiments. *B*, survival after UV-irradiation at 80 J/m^2. *Error bars* represent S.D. calculated using the data of at least four independent experiments.

concentration of DnaE. This raises the possibility that DnaE has an error-prone polymerase activity. To test this property, we analyzed *in vitro* the ability of DnaE to incorporate nucleotides in front of DNA lesions and to extend them, a process termed translesion synthesis (TLS) or replication bypass. TLS was assayed on primed circular ssDNA templates or on primed oligonucleotides containing a single site-specific lesion. Undamaged substrates were used as control. We observed that DnaE was able to bypass *N*2-acetylaminofluorene guanine adduct (G-AAF) located in three different sequence contexts (Fig. 7*A*) (40, 41). Bypass of the $3G_1$-AAF substrate was inefficient (Fig. 7*A*, *lanes 11* and *12*; ~5% bypass) and generated full-length TLS. In the $3G_3$-AAF context, the efficiency of TLS was markedly increased (Fig. 7*A*, *lanes 14* and *15*; up to 20% bypass), and the following two distinct bypass products were generated: a full-length product (TLS 0; ~5%) and a 1 nucleotide shorter product (TLS −1; ~15%). Finally, with a greater efficiency (Fig. 7*A*, *lanes 5* and *6*; ~30% bypass), the following two distinct bypass products were observed in the NarI context: a full-length product (TLS 0; ~5%) and a 2 nucleotides shorter product (TLS −2; ~25%). Although DnaE is able to bypass G-AAF adducts, a marked block one nucleotide before or at the lesion (position L −1 or L0, respectively) was nevertheless observed in all sequence contexts, suggesting that insertions opposite the lesion and/or further extensions are strongly inhibited.

The nature of the nucleotide inserted in front of the G-AAF was determined using single nucleotide incorporation assays. Regardless of the sequence context, we observed that the "correct" nucleotide (*i.e.* C) was preferentially inserted across from the G-AAF adduct (Fig. 7*C*). In the case of $3G_1$-AAF, C represented the major insertion (80%), although some minor insertion events occurred (A 15% and G 5%). Interestingly in the $NarG_3$-AAF context, C was either inserted once (~90%) or twice (10%). The preferential incorporation of the correct nucleotide in front of the G-AAF adduct allows us to explain the nature of the different bypass profiles observed. TLS products observed in $3G_3$-AAF and $NarG_3$-AAF sequence contexts, respectively (Fig. 7*A*), could be explained by a slippage mechanism. Indeed, insertion of C opposite the G-AAF adduct forms a replication intermediate (lesion terminus) that may be present in two distinct conformations, non-slipped and slipped. Elongation from the non-slipped conformation will generate TLS 0 (full-length TLS), whereas extension of the slipped one will produce mutagenic (frameshift) TLS-1 or TLS-2 depending on the sequence context surrounding the lesion. Such slipped conformations have been found to be strongly stabilized by the presence of the AAF adduct (42). *B. subtilis* DnaE seems more proficient to bypass $3G_3$-AAF and $NarG_3$-AAF by a slippage mechanism rather than by direct extension of the G-AAF:C mispair (Fig. 7*A*). The absence of frameshift observed with $3G_1$-AAF template results from the impossibility to form slippage intermediates in this sequence context (Fig. 7*A*) (26, 43, 44).

In contrast to the AAF lesions, DnaE did not bypass neither a benzo-(*a*)-pyrene adducted guanine (G-BaP) nor a (6-4)TT UV photoproduct (Fig. 7*A*, *lanes 16–18* and *19–21*). The polymerase is arrested one nucleotide before the G-BaP adduct (L −1 position) and is blocked in front of the 3′-T of the (6-4)TT photoproduct. Incorporation assays showed that the correct base (*i.e.* A) is preferentially inserted (70% of the incorporation events), G being inserted in the remaining 30% in front of the 3′T (Fig. 7*C*).

We also investigated the ability of DnaE to bypass a non-coding lesion using the abasic site (AP) as a model lesion. DnaE was able to bypass the AP lesion with a poor efficiency generating a TLS product 1 nt shorter (TLS −1) compared with full-length product obtained on the undamaged template (Fig. 7*B*, *bottom*). Single nucleotide incorporation assays showed an insertion of G opposite the AP site (Fig. 7*C*). This insertion allows us to explain the formation of a 1-nt shorter TLS product that may arise by a "dNTP-stabilized misalignment" mechanism (45, 46). Indeed, in the dNTP-stabilized misalignment mode, a transient misalignment occurs before incorporation, with the abasic moiety bulged out of the helical plane. The incoming (incorrect) dNTP is aligned on the next template base, and extension of the intermediate generates a −1 frameshift bypass product.

DISCUSSION

The bulk of DNA synthesis is catalyzed by replicative polymerases of the B and C families in eukaryotes and prokaryotes,

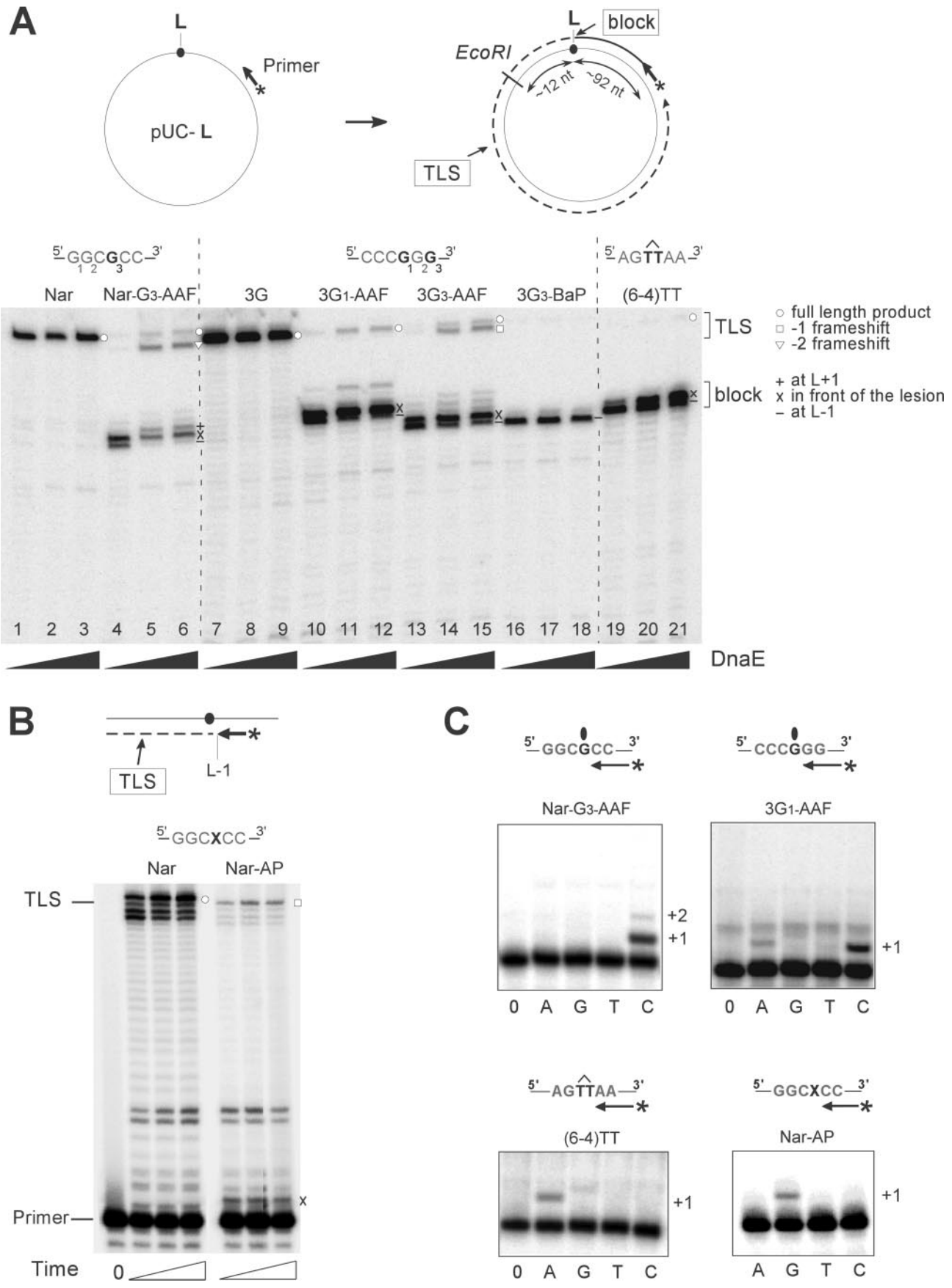

FIG. 7. **Translesion synthesis ability of DnaE on various damaged DNA substrates.** *A*, synthesis past various replication-blocking lesions. *Top*, schematic representation of the substrates and replication products. The relative position of the labeled primer (*starred thick arrow*), the lesion (*black oval*, *L*), and the EcoRI restriction site in a series of pUC-L vectors is drawn. Depending on the lesion, primer extension was either blocked before the lesion (*block at L −1*) or at the lesion without further extension (*block in front of the lesion and block at L +1*) or extended past the lesion (*dashed line*) giving rise to the *TLS* products (full-length products (TLS 0) or frameshift products (*TLS −1* and *−2*)). Replication patterns

respectively. In all the reconstituted replication systems described so far, the synthesis is carried out by two copies of a single replicative polymerase embedded in a highly ordered protein complex termed holoenzyme. Interestingly, this replication scheme might not be universal as follows: (i) two different replicative polymerases were shown to be required for DNA replication in some organisms (exemplified by *S. cerevisiae* and *B. subtilis*), and (ii) an extraordinary diverse group of organisms may code for more than one replicative polymerase. Unfortunately, because of the lack of reconstituted system in these organisms, the exact function of each replicative polymerase at the fork is not known. Here we report an analysis of the *B. subtilis* DnaE replicative polymerase. We first carried out a biochemical analysis to determine its general DNA synthesis properties. We also investigated whether DnaE has a mutagenesis function, as do some polymerases of the B and C families (9, 33, 38).

DnaE and DNA Replication—DnaE on its own fully replicates a primed M13 ssDNA. At 37 °C, the elongation rate reaches 60 nt/s and the processivity 1–55 nt. The polymerization rate is stimulated twice by SSB and can be further increased up to 240 nt/s by incubating the reaction mixture at 47 °C, the optimal growth temperature for *B. subtilis*. DnaE has no 3′ → 5′-exonuclease activity, and its polymerization activity is strongly inhibited when the enzyme encounters an annealed 5′-end. Finally, DnaE has a weak strand displacement activity, stimulated by the concerted action of SSB and the encounter of a 5′-flapping tail.

The speed and processivity of DnaE compares favorably with those of other purified replicative polymerase from Gram-negative (*E. coli*), extreme thermophile (*Aquifex aeolicus* and *Thermus thermophilus*), and Gram-positive (*Streptococcus pyogenes* and *S. aureus*) eubacteria (1, 47–50). It is noticeable that among these enzymes, the α subunit of *E. coli* is the "worst" replicative polymerase. Alone or associated in a three-subunit complex called core, the enzyme is inactive on SSB-coated ssDNA (51). On naked ssDNA, it has a polymerization rate at or below 20 nt/s and a processivity of 11 nt (35, 51). Moreover, it is unable to fully replicate the ssDNA template regardless of the amount of the core or the time used (52). Thus, for achieving the high elongation rate (700 nt/s) and processivity (>50 kb) of the holoenzyme, the α subunit should be stimulated by interactions with other components of the holoenzyme. The stimulating subunits are the ϵ proofreader and the β sliding clamp (1, 35, 53, 54). Protein ϵ stimulates 2–4-fold the elongation rate, whereas β stimulates both elongation (~30-fold) and processivity (>1000-fold). This subunit-dependent stimulation of replicative polymerase might be a general phenomenon as it was observed in other systems (47–50). Moreover, the stimulating agents might be conserved over the evolution as only the holoenzymes containing both the proofreader and the sliding clamp exhibit a velocity and processivity similar to those of the *E. coli* holoenzyme (48, 49).

Like other replicative polymerases, *B. subtilis* DnaE might also need to interact with a proofreader and a sliding clamp for becoming fully active. As expected, *B. subtilis* encodes a canonical sliding clamp (55), and DnaE carries a putative sliding clamp-binding motif (56). Thus, it is likely that the two proteins interact. Indeed, stimulation of processivity, but not the rate of synthesis, by the clamp was recently reported for DnaE of *S. pyogenes*, a *B. subtilis*-related low G + C Gram-positive bacterium encoding both pol C and DnaE (48). Surprisingly, *B. subtilis* does not contain a clear homolog of the *E. coli* proofreader. Yet it encodes several putative 3′ → 5′-exonucleases (57), one of which could be the DnaE proofreader.

Altogether, these results are compatible with the hypothesis that DnaE plays a major role in chromosomal DNA synthesis. Several lines of evidence support the hypothesis that DnaE acts with pol C in the *B. subtilis* holoenzyme, and further suggest that pol C might synthesize the leading strand and DnaE the lagging strand. First, biochemical and yeast two-hybrid studies showed that pol C of *S. pyogenes*, *S. aureus,* and *B. subtilis* interact with and are stimulated by the sliding clamp and the clamp loader, even though they do not copurify with these subunits (48, 49, 58, 59). This supports a role for pol C in leading strand synthesis as the polymerase needs to be tethered onto DNA to ensure rapid, processive, and continuous synthesis of the leading strand. Indeed, genetic analysis showed that depletion in pol C activity causes a strong reduction in plasmid DNA replication that may be due to an inhibition of pol C-dependent leading strand polymerization (7). Although DnaE should also be stimulated by the sliding clamp and clamp loader, protein-protein interactions between the *B. subtilis* DnaE and the sliding clamp and clamp loader have not been detected by a two-hybrid assay (59). Possibly, anchoring of DnaE onto the holoenzyme is more labile than that of pol C. This may make sense if DnaE ensures lagging strand synthesis, a process that might require a distributive mode of action of the replicative polymerase. Two independent observations are consistent with this proposal. First, genetic analysis of plasmid replication intermediates showed a specific defect in lagging strand synthesis upon depletion in DnaE activity (7). Second, strong arrest of DnaE-mediated synthesis occurs *in vitro* upon collision with an annealed 5′-end, an event taking place during completion of each Okazaki fragment.

DnaE and Mutagenesis—Western blot analysis indicated that *B. subtilis* cells contain ~300 copies of DnaE and that only ~80 molecules would be necessary for ensuring optimal cell growth and chromosome replication. Moreover, DnaE synthesis was shown to be induced ~3 times upon treatment with DNA-damaging chemicals. This induction depends on DinR, the repressor of the SOS response in *B. subtilis* (60), and an SOS box was found 74 bp upstream of the start codon of DnaE. Altogether, these results establish that DnaE belongs to the SOS regulon. This remarkable result led us to examine whether DnaE has a role not only in replication but also in mutagenesis. To address this question, we modulated DnaE and pol C levels in the cells. Variation in concentration of both polymerases has no effect on spontaneous mutagenesis, but depletion prevents UV-induced mutagenesis, indicating that

were analyzed after EcoRI cleavage of the products and electrophoresis on denaturing gels. *Bottom,* primer extension reactions showing the ability of *B. subtilis* DnaE to bypass various DNA lesions. The nature of the lesion and its sequence context is indicated *above* the gel. *Nar* and *3G* are the corresponding sequences without any lesion used as control. Regular primer extension reactions were performed using 1 fmol of DNA template and either 1, 5, or 10 fmol of DnaE for 10 min at 37 °C. The 5′-end of the labeled primer (primer 2316) maps 91–93 nt upstream from the lesion, according to the modification introduced. EcoRI restriction of TLS products gives rise to 104-nt products. Boxes and/or TLS products are represented with *open symbols* and *arithmetic signs*, respectively, as indicated on the *right*. *B*, synthesis past an abasic site. A 90-mer oligonucleotide containing a single abasic site (*AP*) located in the NarI site (5′-GGC*X*CC-3′, *X* = AP site) was annealed to a ^{32}P-radiolabeled primer ending immediately upstream from the lesion (position *L* −*1*). *Nar-AP* and *Nar* are the abasic site containing template and the corresponding undamaged substrate, respectively. 1 fmol of DNA template was incubated with 10 fmol of DnaE for 0, 1, 5, and 15 min at 37 °C. High amounts of non-elongated primer results from the use of a 2-fold primer excess over template. *C*, nucleotide incorporation by DnaE at various DNA lesions. 2.5 fmol of DnaE was incubated with 1 fmol of DNA template for 10 min at 37 °C in the absence (*0*) or in the presence of each individual dNTP (100 μM; *A*, *G*, *T*, and *C*), as described under "Experimental Procedures."

both enzymes play a role in this process.

To test whether the DnaE-dependent mutagenicity depends on an error-prone activity of DnaE, we examined the capacity of the purified DnaE protein to bypass DNA lesions. We found that DnaE does not bypass lesions that highly distort DNA ((6-4)TT photoproduct and BaP adducts). In front of the BaP lesion, DnaE is not able to insert any nucleotide, whereas it quite inefficiently inserts 1 nt opposite the 3′T of the (6-4) photoproduct and then stops. Conversely, DnaE has a bypass activity on AAF-adducted guanines and on an abasic site (the TLS activity being much more efficient on AAF adducts than abasic sites). Interestingly, with both lesions, frameshift mutations are mainly generated, indicating that DnaE has a greater ability to extend misaligned primer than to directly extend mispaired 3′ termini.

Interestingly, *B. subtilis* DnaE polymerase TLS activity is quite similar to that of the *E. coli* α subunit. Both enzymes are not able to bypass BaP (61) nor (6-4) photoproducts, inserting mainly an A in front of the 3′T of the latter lesion (62), and they poorly bypass abasic sites generating mainly −1 frameshifts (63, 64). Additionally, both enzymes have a marked preference for misalignment extension rather than for direct extension of mismatches (65). However, it should be noted that DnaE bypasses AAF adduct guanines quite efficiently, whereas this lesion completely blocks the progression of the α subunit (66). This indicates that the *B. subtilis* enzyme has a more relaxed active site than its *E. coli* homolog.

It is well known that the polymerase-associated 3′ → 5′-proofreading exonuclease plays a significant role in mutation avoidance. This holds true for damage-containing templates as shown with the T7 pol and the *E. coli* pol I, pol II, and pol III enzymes (67–70). Thus, if DnaE has a proofreader, it is likely that the DnaE-proofreader complex exhibits a lower bypass capacity than described here for the purified DnaE protein.

Dual Function for DnaE and pol C?—Altogether, this study suggests that *B. subtilis* DnaE and possibly pol C have a function in both DNA replication and DNA repair/mutagenesis. Such a dual activity was observed previously for pol III of *E. coli* (reviewed in Ref. 71) and the two eukaryotic replicative polymerases, pol δ and pol ϵ (reviewed in Refs. 9 and 72). The exact function of DnaE in DNA replication is not clear. As suggested previously (7), it might be part of the holoenzyme ensuring lagging strand synthesis. DnaE likely functions in DNA mutagenesis because it is SOS-inducible, and its depletion prevents UV-induced mutagenesis. The involvement of pol C in repair is suggested by the fact that its depletion causes a weak cell sensitivity to UV irradiation and prevents UV-induced mutagenesis. The observation that depletion of both polymerases inhibits UV-induced mutagenesis can be rationalized in three different models. First, one can speculate that upon depletion of pol C or DnaE, more time is available for excision repair systems to process the lesions. Second, it can also be considered that a high concentration of DnaE and pol C might be required to allow *bona fide* error-prone polymerases (pol Y family) to assist arrested replication forks in bypassing lesions. It is interesting to note here that *B. subtilis* encodes two error-prone polymerases of the Y family that are responsible for most (if not all) of the UV-induced mutations (73).[2] If, as hypothesized here, pol Y-mediated UV-induced mutagenesis strictly depends on high pol C and DnaE concentrations, one can infer that the error-prone and replicative polymerases act in the same pathway and that the decrease in concentration of one replicative polymerase overrides the mutagenic function of the other polymerases. In a third model, we could hypothesize that DnaE and pol C are themselves error-prone polymerases. Yet, because of the clear involvement of pol C in DNA replication, it is unlikely that this enzyme has an error-prone activity. This issue is less obvious for DnaE because the biochemical analysis reported here indicates that its active site might be unexpectedly tolerant for bypassing some lesions. However, DnaE does not fulfill the function of an error-prone polymerase *in vivo* because its overproduction does not increase the rate of spontaneous mutagenesis (as it can be observed for pol Y enzymes (74, 75)),[2] and its biochemical properties (velocity, processivity) are in strong contrast to those of error-prone polymerases. Clearly, more analyses are required to fully understand the fascinating biological functions ensured by the two replicative polymerases in DNA metabolism.

While the preparation of this manuscript was in progress (76),[3] a related *in vitro* study was published by O'Donnell and co-workers with the *S. pyogenes* DnaE homolog (76). In agreement with our results, *S. pyogenes* DnaE was shown to have a higher intrinsic ability to bypass DNA lesions than the *E. coli* α subunit. In addition, *S. pyogenes* DnaE is highly inaccurate in replicating undamaged DNA suggesting that it has an error-prone activity (76). The role of this enzyme in *in vivo* mutagenesis remains to be tested.

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Involvement of DnaE, the Second Replicative DNA Polymerase from *Bacillus subtilis*, in DNA Mutagenesis

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