The essential C-family DnaE polymerase is error-prone and efficient at lesion bypass.

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ABSTRACT

DnaE-type DNA polymerases belong to the C-family of DNA polymerases and are responsible for chromosomal replication in prokaryotes. Like most closely related gram-positive cells, S. pyogenes has two DnaE homologs, PolC and DnaE; both are essential to cell viability. PolC is an established replicative polymerase, and DnaE has been proposed to serve a replicative role. In this report we characterize S. pyogenes DnaE polymerase and find that it is highly error-prone. DnaE can bypass coding and non-coding lesions with high efficiency. Error-prone extension is accomplished by either of two pathways, template-primer misalignment and direct primer extension. The bypass of abasic sites is accomplished mainly through “dNTP-stabilized” misalignment of template, thereby generating (−1) deletions in the newly synthesized strand. This mechanism may be similar to the dNTP-stabilized misalignment mechanism used by the Y-family of DNA polymerases and is the first example of lesion bypass and error-prone synthesis catalyzed by a C-family polymerase. Thus, DnaE may function in an error-prone capacity that may be essential in gram-positive cells but not gram-negative cells, suggesting a fundamental difference in DNA metabolism between these two classes of bacteria.
INTRODUCTION

The replicative DNA polymerase of *E. coli*, the α subunit, is encoded by dnaE and functions with a sliding clamp processivity factor and a clamp loader for efficient chromosome duplication (1-3). Most gram-positive organisms have two essential polymerase subunits with high sequence similarity to *E. coli* α subunit, called PolC and DnaE (4-7). PolC is essential and has long been known to be required for replication of the chromosome (6,8-11). Both PolC and DnaE function with the β sliding clamp and a clamp loader (12).

The dnaE gene is essential in gram-positive bacteria that are closely related to *S. pyogenes*, such as *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus subtilis* (4,5,7). Furthermore, at nonpermissive temperature, a ts *B. subtilis* dnaE mutant is disrupted in lagging strand synthesis but not leading strand synthesis of plasmid DNA (4). These data suggest that PolC and DnaE are both replicative polymerases, with PolC polymerase synthesizing the leading strand and DnaE polymerase synthesizing the lagging strand. Pol C contains an intrinsic 3’-5’ proofreading exonuclease while DnaE, like *E. coli* α, lacks proofreading activity. However, *E. coli* α binds tightly to ε, a small 3’-5’ exonuclease that supplies proofreading function to α (13). *S. pyogenes* also contains a homolog to ε, but whether it associates with DnaE has not been tested.

*E. coli* DNA polymerase III is a high fidelity polymerase that is severely blocked by DNA lesions (1,14). Specialized repair polymerases are required to synthesize across DNA lesions *in vivo* (15). Bypass of DNA lesions, or
“translesion synthesis”, is performed by the Y family of DNA polymerases which are characterized by their ability to bypass lesions in synthetic DNA templates and lack an associated exonuclease (16).

In this report we examine the biochemical properties of the *S. pyogenes* DnaE polymerase and find that it has low fidelity, efficiently misincorporates nucleotides into DNA and extends mismatched termini of primers. DnaE also proficiently extends a primer across an abasic lesion. Moreover, *S. pyogenes* DnaE does not associate with the *S. pyogenes* ε homolog. Thus, *S. pyogenes* DnaE polymerase is an error-prone C-family polymerase and may constitute the first example of an error-prone polymerase that is essential for cell viability.

**EXPERIMENTAL PROCEDURES**

**Materials**- Unmodified oligonucleotides were purchased from IDT. Modified oligonucleotides were purchased from Midland Co. Ultrapure deoxyribonucleotide triphosphates and ATP were purchased from Amersham Biosciences. Oligonucleotides were labeled with \[^{32}\text{P}]\text{ATP}\) (Perkin Elmer Life Sciences, Inc) and phage T4 polynucleotide kinase (New England Biolabs). *Streptococcus pyogenes* DnaE, PolC, SSB, β, and τδδ’, were cloned, overexpressed in *E. coli*, and purified to homogeneity, as described (12). The *E. coli* core (exo-) complex was constituted from α, θ, and an ε mutant (Asp12Ala, Glu14Ala) as previously described (17).
Purification of \textit{S. pyogenes} exonuclease encoded by DnaQ - A search of the \textit{S. pyogenes} genome database using the sequence of several known \textit{dnaQ} proteins yielded a weak homolog of 26\% identity. PCR primers were designed as follows: The upstream 31-mer, 5' - GCG AAA TAA TCA CAA CAT ATG ACG AAG GCC C- 3', contains a NdeI site, and the downstream 29-mer, 5' - GAC CTA GCG GGA TCC GCA AGG GCT AAC CC- 3' contains a BamHI site. A PCR product obtained with these primers was digested with NdeI and BamHI, purified (624 bp) and ligated into pET 11a vector to produce pET11a.SpDNA. The \textit{S. pyogenes} DnaQ gene encodes a 208 residue protein of 23,176 Da.

The pET 11a.SpDNA plasmid was transformed into \textit{E. coli} BL21(\lambda DE3)\textit{recA}. A single colony was used to inoculate 12L LB broth supplemented with 200 \(\mu\)g/ml ampicillin. Cells were grown at 37°C to OD\textit{\textsubscript{600}}=0.5 at which point the temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs at 15°C, then cells were collected by centrifugation, resuspended in 50 mM Tris-HCl, pH 7.5, 10\% sucrose, 1M NaCl, 30 mM spermidine, 5 mM DTT. Cells were lysed by two passages through a French press (15,000 psi) followed by centrifugation at 13,000 rpm for 30 min. Ammonium sulfate was added to the lysate to a final concentration of 0.226 g/ml. The precipitate was collected by centrifugation and the protein pellet was resuspended in buffer A (20 mM Tris-HCl (pH 7.5), 4\% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol (DTT)) and then dialized against buffer A. The dialyzed protein (250 mg) was applied to a 30 ml Fast Flow Q column and then eluted with a linear gradient of 50-500 mM NaCl in Buffer A. The peak fractions (fr 27-42; 121 mg) were pooled, dialyzed and applied to a 15 ml Heparin-Affigel.
column and eluted with a linear gradient of 50-500 mM NaCl in Buffer A. The peak fractions were pooled (30 mg), dialyzed against buffer A containing 50 mM NaCl and then one-half was applied onto a 1 ml MonoQ column. The MonoQ column was developed with a linear gradient of 50mM - 500 mM NaCl in Buffer A. The peak fractions were pooled and stored frozen at – 80°C.

**DNA substrates** - Studies of insertion kinetics utilized a 50mer template (5’-CCAACGACAGCAGTAATCANGCCAGGAAGCTGTGCGCGCGGTCC T -3’), where N is A, T, C, G, X-abasic, 2-aminopurine, or 8-oxo-dG annealed to 5’-32P labeled 30 mer (5’- AGGACCGCAGGCACAGCTTCTCTGGCTGGC -3’). For mismatch extension kinetics the same 50mer template strand (with no lesion) was annealed to a 5’-32P labeled 30mer as above except the 3’ nucleotide was either A, T, C, or G. For studies on extension past an abasic site the 50mer template contained an abasic site at position 20 (from the 5’ end), and 5’-32P labeled 31mer (5’- AGGACCGCAGGCACAGCTTCTCTGGCTGGC -3’, where N is A, T, C, or G) was annealed to it. For replication on homopolymer DNA, the following synthetic DNA oligonucleotides were used: “Template (T)” [5’-(T)20TTAGCCAGGAAGCTTGGCCGCGCGGTCTCCCTGTGCTGGC -3’] or “Template (TC)” [5’-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATTGACCCAGGAAGCTTGGCCGCGCGGTCTCCCTGTGCTGGC -3’] were annealed to 5’-32P labeled “Primer (A)” [5’-AGGACCGCAGGCACAGCTTCTTGCAAAA -3’]. “Template (C)” [5’-(C)20CCCAGCCAGGAAGCTTGGCCGCGCGGTCTCCTGTGCTGGC -3’] was annealed to 5’-32P labeled “Primer(G)” [5’-AGGACCGCAGGCACAGCTTCTGGCTGGC -3’].
**Primer extension reactions** - Primer extension reactions contained 3.2 nM $^{32}$P labeled primer-template, 16.9 nM of DNA polymerase (S. pyogenes DnaE, S. pyogenes PolC, or E.coli core (exo-)), 500 μM each of the four dNTPs in 25 μl of 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 40 μg/ml BSA, 8 mM MgCl$_2$. Where indicated, reactions contained either 10 ng or 50 ng S. pyogenes DnaQ. Reactions were incubated for 5 min at 37°C, before being quenched with an equal volume (25 μl) of 96% formamide/40 mM EDTA/0.001% bromophenol blue/0.001% cyanol blue.

**Exonuclease activity assays** - Exonuclease assays contained 3.2 nM of a 5’-labeled 30mer DNA oligonucleotide and 100 ng of S. pyogenes DnaQ in 25 μl of 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 40 μg/ml BSA, 8 mM MgCl$_2$. Reactions were incubated at 37°C and aliquots of 25 μl were removed at the times indicated. Reactions were quenched with an equal volume (25 μl) of 96% formamide/40 mM EDTA/0.001% bromophenol blue/0.001% cyanol blue.

**Steady-state kinetics** - Reaction conditions were optimized for reaction times and enzyme concentration, such that less than 20% of the primed template was extended. Several time courses were performed to determine the range over which nucleotide insertion was linear. Reactions contained 6.4 nM $^{32}$P labeled primer-template in 5 μl of Reaction Buffer (40 mM Tris-HCl (pH 7.5), 8% glycerol, 0.2 mM EDTA, 10 mM DTT, 80 μg/ml BSA, 16 mM MgCl$_2$). Primer-template mixtures were incubated with various concentrations of DnaE (0.033 nM-16.9 nM) for 1 min at 37°C, then synthesis was initiated by mixing with an
equal volume of dNTP buffer (Reaction buffer containing dNTPs). To measure nucleotide insertion, the polymerase-primer-template mixture (5 µl) was added to 5 µl of dNTP buffer containing various concentrations of correct nucleotide (0.03 µM-1.2 mM) or incorrect nucleotide (0.01 mM-20 mM). To measure mismatch extension kinetics the concentration of TTP was 0.03 µM-1.2 mM for correct primer-template termini and 0.01 mM-20 mM for incorrect primer-termini. Reactions were incubated at 37°C for 1-10 min and were quenched with a 20µl solution of 96% formamide / 40 mM EDTA / 0.001% bromophenol blue / 0.001% cyanol blue. Reaction products were resolved on a 12% polyacrylamide gel (8 M Urea), exposed to a PhosphorImager screen and analyzed using ImageQuant software. Apparent \(V_{max}/K_m\) values were determined as described (18-20). The intensity of the bands corresponding to the starting primer (\(I_0\)) and the primer extended by one nucleotide (\(I_1\)) were used to calculate velocities. Relative velocity was calculated using \(v = 100(I_1/(I_0+0.5I_1)t)\). Apparent \(V_{max}\) and \(K_m\) values were determined from a Hanes-Woolf plot of the data. A Hanes-Woolf plot of \([dNTP]/v\) versus \([dNTP]\) was fitted by linear least-squares to obtain the intercept (\(K_m/V_{max}\)) and slope (\(1/V_{max}\)). The nucleotide misincorporation efficiency \(f_{inc}\) and intrinsic mismatch extension frequency \(f_{ext}^o\) are expressed as ratios: \((V_{max}/K_m)w/(V_{max}/K_m)r\), where \(w\) and \(r\) refer to either correct (r) or incorrect (w) deoxyribonucleoside pair formation.

**Analysis of DnaE and DnaQ by Gel Filtration**- Gel filtration analysis of protein mixtures was performed using an HR 10/30 Superose 6 column (Perkin Elmer Life Sciences, Inc) equilibrated with buffer A containing 100 mM NaCl. Protein
mixtures were incubated at 25°C for 10 min, and then the sample was injected onto the column. After the first 6.6 ml, fractions of 170 µl were collected. Fractions were analyzed in 10% SDS-polyacrylamide gels. Analysis of DnaE and DnaQ subunit was performed using either 800 µg DnaE alone, 400 µg DnaQ alone, or a mixture of both proteins (800 µg DnaE and 400 µg DnaQ) in 200 µl of buffer A containing 100 mM NaCl.
RESULTS

*S. pyogenes* DnaE is a low fidelity polymerase - To examine the fidelity of the *S. pyogenes* DnaE polymerase, DnaE was incubated with a synthetic primer-template, but in the presence of only one dNTP. Surprisingly, *S. pyogenes* DnaE polymerase incorporated dNTP in each case, and in fact extended the primer up to 7 bases (Figure 1A). We examined the *S. pyogenes* DnaE preparation for terminal transferase activity, but found no activity in the absence of a primed site (data not shown). As controls, we examined *E. coli* core (exo-) and *S. pyogenes* PolC at the same concentrations as *S. pyogenes* DnaE. *E. coli* core (exo-) contains α, θ, and a mutant ε subunit, in which two residues are replaced by alanines to eliminate exonuclease activity. The results indicate that these polymerases appear to be less efficient in incorporation of a mismatched dNTP (Figure 1B and 1C, all lanes except 1, 6, 11, and 16). In fact, unlike *S. pyogenes* DnaE, neither *E. coli* core (exo-) nor *S. pyogenes* PolC can extend the primer to the end of the template in presence of all four dNTPs. *S. pyogenes* PolC has an intrinsic exonuclease activity which accounts for the degradation products observed in Fig. 1C. Overall, these observations suggest that *S. pyogenes* DnaE has quite low fidelity, especially compared to established replicative polymerases.

The above experiments were performed under conditions using excess polymerase and a long incubation time. Next, we performed a similar experiment except under single hit conditions, accomplished using a low enzyme concentration such that less than 20% of the primers were extended, thereby ensuring that primer template had a productive encounter with the polymerase
only once (18). The first unpaired template residue and the incoming dNTP were systematically varied. The concentration of incoming dNTP was titrated in separate reactions providing the steady state kinetic data necessary to determine the apparent $V_{\text{max}}/K_m$ and the efficiency of misincorporation (Table I, template residues A, T, C, and G) (18-20). Consistent with the results of experiments in Figure 1, *S. pyogenes* DnaE misincorporates dNTP at a high rate ($f_{\text{inc}}$ 1.4 x 10$^{-3}$ to 5.8 x 10$^{-2}$). These misincorporation rates are 100-1000 fold higher than those reported for replicative polymerases, such as $\alpha$ subunit from *E. coli* (10$^{-4}$ to 10$^{-5}$) (21). These rates are also higher than those reported for error-free repair polymerases, such as Pol II (misincorporation efficiency ~10$^{-4}$) (22-24).

**S. pyogenes** DnaE extends mismatches at a similar efficiency as misincorporation - The experiments in Figure 1 and Table I demonstrate that *S. pyogenes* DnaE has an impressive capacity to misincorporate dNTPs, thus forming a DNA mismatched 3’ primer terminus. Next we studied DnaE action on DNA substrates that contain a preformed mismatch at the 3’ primer terminus (Figure 2A). In the presence of all four dNTPs *S. pyogenes* DnaE efficiently extended almost all of the mismatched primer to near the end of the template (Figure 2A, lane 1). In contrast, the replicative *E. coli* core (exo-) polymerase could not extend the mismatched primer at all under identical conditions (Figure 2A, lane 6). These results are consistent with those of previous reports which demonstrate that neither *E. coli* DNA polymerase III (exo-) nor isolated $\alpha$ subunit extend mismatches (25-27).
The *S. pyogenes* DnaE often stops one nucleotide short of the end of the template (lane 1). This observation indicates that mismatch extension on this substrate appears to proceed primarily by template-primer misalignment. This observation is further supported by the efficient incorporation of dTTP in lane 3, which may be explained by “flip-out” of the G in the template strand, thereby pairing the 3’A to the T in the template. This can also explain lack of primer extension in lanes 4-5, since there are no flip-out solutions for dC or dG. Lane 2 shows that dATP can also be incorporated efficiently by direct extension of the mismatched terminus.

To examine the mismatch extension frequency of *S. pyogenes* DnaE polymerase in greater detail, the primer terminus and "paired" template nucleotides were each systematically varied to create 4 correctly paired and 12 mismatched primer templates (Figure 2B). Only the correct incoming dTTP was included, and conditions were optimized such that less than 20% of the primer was extended. Mismatch extension frequency was calculated by titrating dTTP into different reactions and determining $V_{\text{max}}/K_m$ from the resulting time courses, as described (18,20). Of the 12 mismatches, the frequencies of mismatch extension varied considerably ($1.3 \times 10^{-1}$ to $8.4 \times 10^{-5}$), but were generally high compared to estimates of mismatch extension frequencies by *E. coli* core (exo-) ($>10^{-5}-10^{-6}$) (21,25,27). In contrast, mismatch extension by error-prone polymerases occurs over a range of $10^{-1}-10^{-2}$ for Pol κ, Pol ζ, and Rev1, and to $10^{-4}-10^{-5}$ for Pol IV (28-30).

Figure 2B shows that four mismatched primed templates have high frequencies of mismatch extension (between $1.3 \times 10^{-1}$ and $1 \times 10^{-2}$), and the other eight have more moderate efficiencies (between $1 \times 10^{-2}$ and $8.4 \times 10^{-5}$). The four
mismatched primed templates that have high extension efficiencies are pyrimidine/purine mismatches, and the eight with moderate extension efficiencies are purine/purine or pyrimidine/pyrimidine mismatches. The purine/pyrimidine mismatches may form a better steric fit within the enzyme active site, enabling DnaE to extend the mismatch more readily. The same pattern of mismatch extension has been shown for Pol IV polymerase (28). These may be compared with previous studies of *E. coli* core and α subunit in which mismatch extension efficiency is exceedingly poor (10⁻⁶) (21,25,27).

In general, repair polymerases typically extend mismatches at a similar rate at which they incorporate mismatched nucleotides (14,15). One exception to this rule is pol ζ which extends mismatches more frequently than it misincorporates them, and thus it has been proposed that the *in vivo* role of pol ζ is to extend from mismatches (30). HIV reverse transcriptase has also been shown to extend mismatched primer termini with high efficiency (31,32). In Figure 2C, mismatch extension frequency is plotted versus misincorporation frequency for *S. pyogenes* DnaE. If the frequencies are equal, the points should lie on the diagonal line bisecting the graph. However, most of the points lie near or below this diagonal line. Thus, DnaE is not more efficient at mismatch extension relative to misincorporation.

**Incorporation specificity at an abasic site** - Formation of abasic lesions in the chromosome is a fairly common event. An abasic site is considered "noncoding", since the original base information is lost (33-35). Early studies suggested that the preferred dNTP incorporated opposite an abasic site followed
the A-rule, where dATP is preferentially inserted opposite an abasic site (36). Recent studies have revealed that some polymerases do not follow the A-rule (15,30,37,38). Instead, some of these polymerases skip over abasic lesions, thereby generating (-1) deletions. This mechanism is referred to as dNTP-stabilized template misalignment (28,37).

We investigated the response of *S. pyogenes* DnaE polymerase upon encountering an abasic site. In Figure 3A, *S. pyogenes* DnaE was incubated with a primed template containing an abasic site and all four dNTPs. The results show that *S. pyogenes* DnaE is capable of extending most of the primers past the abasic site to the end of the template, although the product analysis shows that it pauses at the abasic site. *E. coli* core (exo-) was incapable of inserting a dNTP opposite the abasic site. *S. pyogenes* PolC, which contains an intrinsic 3'-5' exonuclease activity, degraded the primed template rather than extend it. The intrinsic ability of *S. pyogenes* DnaE to extend a primer past the abasic site suggests that it may function in some bypass capacity.

We next asked whether *S. pyogenes* DnaE skips over the abasic lesion (like Pol IV), or whether it incorporates a specific dNTP opposite the abasic site (like Pol II). If DnaE skips over the abasic site, the final length of the extended primer should be at least one nucleotide shorter than the template strand. Results in Figure 3B shows that most of the fully extended primer strand is in fact one to two nucleotides shorter than the template strand. Next we asked whether DnaE can use any dNTP to extend the primer terminus positioned at an abasic site. The results in lanes 2-5 of Figure 3B demonstrate that *S. pyogenes* DnaE can utilize dA, dT and dG. Incorporation of dT is likely due to dNTP-stabilized template misalignment, by pairing with the A just beyond the abasic site.
Incorporation of dA may be the result of direct misincorporation following the A-rule. However, it would appear that DnaE does not only follow the A-rule, as dGTP is also incorporated, and even dCTP to some extent. In fact, multiple incorporation events are observed in the presence of either dCTP or dGTP. We then included only one dNTP in a reaction under single hit conditions for single nucleotide extension as in Table I. Efficiency of nucleotide incorporation was calculated for each dNTP with the result that all dNTPs are incorporated with high frequency (Table I). dTTP is incorporated with a much higher efficiency compared to the other dNTPs, consistent with the results of Figure 3B discussed above.

*S. pyogenes* DnaE does not skip over a template base mismatch - To further examine the properties of DnaE, we constructed a set of primer-templates with well-defined homopolymeric extensions (Figure 4). *S. pyogenes* DnaE efficiently extends the primer to the end of a homopolymeric dT template in the presence of all four dNTPs (Figure 4A, lane 1). The primer is not extended very far if only an incorrect dNTP is added (lanes 3, 4, 5). The one or two nucleotides incorporated in lanes 3-5 are most likely due to misincorporation. Interestingly, *S. pyogenes* DnaE did not reach the end of the template when only dA was added to the reaction (lane 2), suggesting that slippage on the template occurs. We presume that in the presence of all four dNTPs, the template has time to realign into proper register when the polymerase binds an incorrect dNTP, and that this action prevents slippage products. Specifically, in the presence of all four dNTPs, one of the three non-cognate dNTPs will often occupy the active site and
will need to diffuse back out which may buy sufficient time for the template to realign.

Next we examined DnaE on a homopolymeric dC template (Figure 4B). In the presence of all 4 dNTPs, or dGTP (lanes 6 and 10, respectively), DnaE extends the primer to either full length or 1 nucleotide shy of full length, indicating that some slippage occurs whether all 4 dNTPs are present or not. dTTP and dCTP are misincorporated on this template with similar efficiency as dATP, supporting the conclusion that DnaE directly misincorporates nucleotides with high efficiency (lanes 7, 8, 9).

Next we designed a template that is homopolymeric in T (similar in sequence to that in Figure 4A) but is interrupted at two positions with dC (Figure 4C). *S. pyogenes* DnaE still extends the primer to the end of this template in the presence of all four dNTPs (Figure 4C, lane 11). However, if only dATP is added, two dark bands are visible, one representing a primer extension product of 6 nucleotides, the other a primer extension product of 12 nucleotides (lane 12). The C residues are positioned 6 and 12 bases from the primer terminus. Thus the *S. pyogenes* DnaE incorporates a dATP opposite the C residue at each position and then pauses before proceeding (Figure 2B). It remains possible that instead of directly incorporating dATP opposite C, DnaE may flip-out the C residue and incorporate dATP at the next T, followed by template realignment to form a mismatched 3’ terminus, the extension of which causes the polymerase to pause. Furthermore, there is a light band representing full primer extension to the end of the template, confirming that no bases are skipped (lane 12).
**S. pyogenes DnaE efficiently bypasses 8-oxo-dG and 2-AP bases** - We next determined how *S. pyogenes* DnaE responds to the lesion, 8-oxo-dG (8-oxo-7, 8-dihydro-2'-deoxyguanosine) (Figure 5). 8-oxo-dG is a common DNA lesion that occurs as a product of oxidative damage, and exists in both *syn* and *anti* conformations (39). In the presence of all four dNTPs, *S. pyogenes* DnaE extends essentially all the primer past the 8-oxo-dG lesion (Figure 5, lane 1). It also efficiently misincorporates bases opposite 8-oxo-dG in each of the single nucleotide addition reactions (lanes 2-5). A comparison with the *E. coli* core (exo-) is shown in Figure 5B. Results using individual dNTPs in Figure 5 show that DnaE can utilize any of the four dNTPs to extend the primer past 8-oxo-dG, and does so more efficiently than *E. coli* core (exo-).

In the favored *syn* conformation, 8-oxo-dG forms base mispairs with dA. This mispair contributes significantly to mutation rates in oxidatively damaged cells by generating G:C to T:A transversion mutations. In the *anti* conformation, 8-oxo-dG correctly pairs with dC. Results in Table I demonstrate that *S. pyogenes* DnaE incorporates both dA and dC with similar high efficiency. These results suggest that *S. pyogenes* DnaE can repair genomic DNA that has suffered oxidative damage, but in a potentially mutagenic fashion.

We next examined the response of *S. pyogenes* DnaE to the nucleotide analog 2-aminopurine (2-AP). 2-AP is a strong genotoxic mutator, which efficiently induces the SOS response in prokaryotic cells and is also highly toxic to eukaryotic cells (40,41). *S. pyogenes* efficiently bypasses the 2-AP nucleotide analog, extending all the primers in the presence of the four dNTPs (Figure 5, lane 6).
2-AP is predominantly incorporated opposite T. However, in subsequent steps of replication 2-AP can mispair with C, thereby generating A:T to G:C transitions. *S. pyogenes* DnaE incorporates either dT or dC opposite 2-AP with similar high efficiency (Table I), consistent with the well established mutagenic potential of 2-AP (42-44). Results in Figure 5 (lanes 7-10) demonstrate that dT is efficiently incorporated opposite 2-AP by *S. pyogenes* DnaE, and the other dNTPs are incorporated to some extent (Table I). Thus, *S. pyogenes* DnaE can bypass several different DNA lesions and nucleotide analogs in a potentially error prone manner. *E. coli* core (exo-) incorporates nucleotides much less efficiently opposite the same lesion (Figure 5, lanes 11-20).

**Lack of interaction of DnaE with the DnaQ homolog** - The *E. coli* ε exonuclease subunit (encoded by dnaQ) interacts tightly with the α polymerase (product of the dnaE) of DNA polymerase III (45). This 3’-5’ exonuclease subunit increases the fidelity of the *E. coli* DNA polymerase III and further diminishes its ability to bypass lesions (21,26). Since *S. pyogenes* DnaE polymerase is a homolog of the *E. coli* α subunit, we reasoned that perhaps DnaE polymerase and the DnaQ homolog of *S. pyogenes* might also interact, like *E. coli* α and ε, thus providing proofreading function to DnaE.

To test this idea, we identified, cloned, overproduced, and purified the *S. pyogenes* DnaQ homolog. First we examined *S. pyogenes* DnaQ for exonuclease activity by incubating DnaQ with a 5’-labeled 30mer DNA oligonucleotide. The results, in Figure 6A, show disappearance of the full length 30mer DNA and
appearance of the degradation ladder, indicating presence of the 3’-5’
exonuclease activity.

To test physical interaction between *S. pyogenes* DnaE and DnaQ, we
incubated the two proteins, and then analyzed them for complex formation using
a gel filtration sizing column. The results in Figure 6B, show that *S. pyogenes*
DnaQ does not coelute with DnaE, indicating that they do not interact. Although
*E. coli* αε complex is stable, and can be isolated by gel filtration, it is possible that
*S. pyogenes* DnaE and DnaQ interact, but that the complex is too weak to detect
by this technique. Therefore we studied *S. pyogenes* DnaQ for an effect on the
DNA polymerase activity of DnaE.

The ability of *E. coli* α to bypass a lesion is inhibited by ε (46,47). To
examine whether *S. pyogenes* DnaQ affects lesion bypass activity of DnaE, we
incubated DnaE and DnaQ on a template containing an abasic site. Figure 6C
shows that DnaQ neither stimulates nor inhibits DnaE-catalyzed DNA synthesis.
The result is consistent with inability of DnaQ to interact with DnaE.
DISCUSSION

*S. pyogenes* DnaE is a promiscuous polymerase - *S. pyogenes* DnaE and *E. coli* α subunit share 34% sequence identity and 47% sequence similarity. Despite this high degree of sequence similarity the two proteins exhibit markedly different biochemical properties suggesting that the proteins serve different functional roles *in vivo*. *E. coli* α is known to be the primary replicative polymerase, and its biochemical properties are typical of a protein designed for replication: high fidelity synthesis of undamaged DNA templates, poor ability to bypass DNA lesions, and poor ability to extend from DNA mismatches (1,14). Moreover, the replicative polymerase binds to a proofreading subunit, ε, which further enhances fidelity and retards its ability to bypass lesions. Finally, the replicative polymerase binds tightly to a ring-shaped clamp, which enhances the processivity of the polymerase by preventing it from falling off the DNA (2,3).

The current study demonstrates that *S. pyogenes* DnaE does not share the biochemical properties of a replicative polymerase. In contrast, *S. pyogenes* DnaE has biochemical properties that are typical of a repair polymerase: low fidelity synthesis of undamaged DNA templates, efficient bypass of DNA lesions, and efficient extension from DNA mismatches. Furthermore, DnaE polymerase does not bind to the *S. pyogenes* DnaQ homolog of the *E. coli* ε proofreading subunit encoded by *dnaQ*. However, we cannot exclude the possibility that *S. pyogenes* DnaE might bind DnaQ *in vivo*, perhaps through a third bridging subunit that has yet to be identified. *E.coli* core contains a third subunit, θ. However, α binds
ε directly and θ does not serve this role (48,49). Further, θ is dispensable in E. coli (50) and homologs of θ do not appear to be present in many organisms.

There are two different paths a polymerase may take to bypass an abasic lesion: misincorporation opposite the abasic site, catalyzed by Pol II and Pol V, or skipping the abasic site by dNTP stabilized template misalignment, catalyzed by the Y-family polymerases, Pol IV (DinB) and Pol k. DnaE prefers to skip over an abasic site (Figure 7). On undamaged templates DnaE most likely utilizes a combination of direct misincorporation and template-primer misalignment to extend either a mismatched or a correctly paired primer (Figure 7). The mechanism of misalignment could be either dNTP-stabilized or misincorporation misalignment (51).

**Does DnaE function at the replication fork?** The current study demonstrates that S. pyogenes DnaE is an error-prone polymerase. It seems unlikely that an error-prone polymerase would participate in bulk chromosomal synthesis. Perhaps DnaE is more accurate in vivo, but it is also possible that it may function in another capacity. For example, DnaE may serve to move a stalled replication fork past a blocking lesion. When a replication fork encounters a DNA lesion, the replication apparatus is expected to stall, and repair proteins must be recruited to correct or bypass the lesion before replication can be restarted. An example of this type of action in E. coli is promoted by Pols IV and V. E. coli Pol IV and Pol V are induced as part of the SOS response, and they function to bypass lesions in an error-prone fashion (28,38). These polymerases are not essential to E. coli, and in fact most lesions are either repaired before the
replication fork encounters them, or are avoided by replication restart or recombinative repair rather than bypassed by an error-prone DNA polymerase (52-55). Gram-positive cells also contain Y-family DNA polymerases. However, the fact that DnaE is essential, at least in most gram-positive cells examined to date, may suggest that some blocks to replication occur frequently in gram-positive cells and DnaE is needed to circumvent them.

Like Pols IV and V in E. coli, S. pyogenes DnaE may also be a participant in the SOS response in gram-positive bacteria. The S. pyogenes dnaE gene contains a putative SOS response regulon in its sequence, a dinR binding box (data not shown) (56). Furthermore, in the distantly related species M. tuberculosis, transcription of dnaE is stimulated 14-fold during the SOS response (57,58). Thus, S. pyogenes DnaE may catalyze translesion synthesis in gram-positive cells during the SOS response.

Translesion synthesis by S. pyogenes DnaE is probably quite costly to the organism, as the current study demonstrates that it is highly error-prone. Thus DnaE action on undamaged templates may also result in frequent base substitutions or (-1) frameshifts. If this is the case for DnaE, there must be some mechanism to harness its action, directing it only as needed to bypass blocks to replication that are not solved by error-free processes.

**How can a polymerase in the C family replicative polymerase class function as a sloppy polymerase?** - Studies of polymerase fidelity suggest that the size and the geometry of the active site of polymerases correlate with the fidelity of the polymerase (14,59). Generally, polymerases with tight active sites are able to
accommodate only correctly paired bases, conferring high fidelity (60,61). DNA polymerases with large flexible active sites have sufficient extra space to accommodate DNA lesions and thereby may bypass the lesion, but at a cost to fidelity because the loose active site does not provide exquisite base discrimination. Thus, one may expect that S. pyogenes DnaE may have a larger active site compared to PolC or E. coli α subunit. However, it has also been shown that polymerase fidelity can be drastically lowered by simply mutating a few residues around the active site. A good example of this point is a mutation study of the active site of the Thermus aquaticus DNA polymerase I (62). Therefore, it is also possible that the differences between PolC and DnaE active site architecture will be subtle.

**Why is an error-prone polymerase essential for cell viability?** - Several studies in closely related gram-positive bacteria have demonstrated that the dnaE gene is essential for viability (5,7). Further, studies by Dervyn et. al. suggest that DnaE may be responsible for lagging strand synthesis although alternative explanations may exist (4). The low fidelity of DnaE and slow speed (60 nt/s), even with the sliding clamp present, are not consistent with the fidelity and speed (1Kb/s) required for chromosomal DNA replication. It is still possible that DnaE functions directly in replication, but if so one may expect that other factors increase its speed and fidelity. We have examined the effect of the clamp loader and β clamp on DnaE, but they had no effect on its ability to bypass an abasic site or extend a mismatch (data not shown).
Why would an error-prone polymerase be essential to the cell? Perhaps, at some phase of the cell cycle an error-prone process is absolutely required. However, the catalytic activity of a DNA polymerase is not always the defining essential activity, as demonstrated in the case of *E. coli* DNA polymerase I and yeast DNA polymerase ε. The polymerase activity of *E. coli* DNA polymerase I is not essential, but the unique 5’-3’ exonuclease activity is essential, being required to remove the RNA from the ends of Okazaki fragments (63). Eukaryotic Pol ε is essential, but one report on this subject indicates that the region encoding the polymerase activity is dispensable (64). Despite these arguments DnaE could still be the lagging strand polymerase and thus reflect major differences and requirements between gram-positive and gram-negative cells. In that case, it will be interesting to see what protein-protein interactions target DnaE to the lagging strand.

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FIGURE LEGENDS

Fig. 1. *S. pyogenes* DnaE polymerase efficiently misincorporates dNTPs into undamaged DNA. DNA polymerases were incubated with \(^{32}\text{P}\) 5′ labeled primer-template (*30mer/50mer) in the presence of either a single dNTP (as indicated) or all four dNTPs for 5 min. The 3′ nucleotide on the primer was varied (A, T, C, or G) as indicated at the top of the figure. Equimolar amounts of each DNA polymerase were used for each reaction. A) *S. pyogenes* DnaE, B) *E. coli* core (exo-), C) *S. pyogenes* PolC. Products of each reaction were resolved on a 12% polyacrylamide (8M urea) gel.

Fig. 2. *S. pyogenes* DnaE efficiently extends mismatched primer termini.

A) Efficiency of extension of a G:A mismatch is compared in reactions performed with *S. pyogenes* DnaE (lanes 1-5) and *E. coli* core (exo-) (lanes 6-10). Reactions contained \(^{32}\text{P}\) labeled primer-template and equimolar amounts of either *S. pyogenes* DnaE or *E. coli* core (exo-). Proteins were incubated for 5 min. B). Intrinsic mismatch extension efficiencies (\(f_{\text{ext}}^{0}\)) were determined for all 16 primer template combinations. \(f_{\text{ext}}^{0}\) is the ratio of \((V_{\text{max}}/K_m)_{\text{mismatch}}/(V_{\text{max}}/K_m)_{\text{correct}}\) pair. C). DnaE is more efficient at misincorporation (\(f_{\text{inc}}\)) than at mismatch extension (\(f_{\text{ext}}^{0}\)). The incorporation data are from Table I and the mismatch extension data from panel B are plotted against one another. Data points which fall above the line indicate more efficient mismatch extension. Data points which fall below the line indicate more efficient misincorporation.
Fig. 3. Efficiency of lesion bypass by *S. pyogenes* DnaE. A) Time course of lesion bypass synthesis performed by *S. pyogenes* DnaE, *E. coli* core (exo-), *S. pyogenes* PolC. DNA products were resolved on a 12% polyacrylamide (8M urea) gel. B) Ability of *S. pyogenes* DnaE polymerase to incorporate individual nucleotides opposite an abasic site. DnaE polymerase was incubated with $^{32}$P labeled primer-template (*30mer/50mer) in presence of either a single dNTP or all four dNTPs. Products were resolved on a 12% polyacrylamide (8M urea) gel. The full length marker (FL) is a 5’-labeled 50mer template strand.

Fig. 4. Error-prone synthesis by *S. pyogenes* DnaE on homopolymer templates. DnaE polymerase was incubated with $^{32}$P labeled primer-template (*30mer/50mer) in presence of either a single dNTP or all four dNTPs for 5 min, and then reactions were quenched. Products were resolved on a 12% polyacrylamide (8M urea) gel. Primer extension reactions were performed on primer templates having 5’ homopolymer extensions of either: A) poly (T) (Lanes 1-6), B) poly (dC) (lanes 7-12), or C) a poly (T) extension containing 2 dC residues at position 6 and 12 (Lanes 13-18). Arrows on the left side of the gel (Panel B) indicate the position of the 2 C’s on the template. For each panel, the arrows to the right indicate the positions of the unextended and fully extended primer. Lanes 6, 12, and 18 show the unreacted primed template.

Fig. 5. *S. pyogenes* DnaE efficiently synthesizes past 8-oxo-dG and 2-aminopurine. DNA synthesis past 8-oxo-dG and 2-aminopurine lesions by *S. pyogenes* DnaE (panel A) and *E. coli* core (exo-) (panel B). Reactions were
performed as indicated in “Experimental Procedures”. DNA polymerases were incubated with $^{32}$P labeled primer-template (*30mer/50mer) in the presence of either a single dNTP or all four dNTPs. Products were separated on a 12% polyacrylamide (8M urea) gel. Identity of a modified base in the template (N is 8-oxo-dG or 2-AP) is indicated at the top of the figure.

**Fig. 6.** The *S. pyogenes* DnaQ homolog does not stably bind DnaE or effect lesion bypass activity. A) Exonuclease activity assay of DnaQ on a 5' $^{32}$P labeled 30mer. B) Interaction between DnaE and DnaQ was analyzed by gel filtration as described in “Experimental Procedures”. Column fractions, indicated at the top of the gel, were analyzed in a 10% SDS-polyacrylamide gel stained with Coomassie Blue. C) Effect of DnaQ exonuclease on lesion bypass synthesis performed by DnaE polymerase. Proteins were incubated in presence of $^{32}$P labeled primer-template (30mer/50mer). A time course of synthesis was performed as indicated in “Experimental Procedures”. Products were separated on a 12% polyacrylamide (8M urea) gel. X indicates the position of the abasic site on the template strand.

**Fig. 7.** *S. pyogenes* DnaE performs direct misincorporation and “dNTP-stabilized” misalignment. Possible products generated by *S. pyogenes* DnaE during error-prone DNA synthesis. A). Synthesis of undamaged DNA can result in either misincorporation (base substitution) or flip-out of a mispaired base and subsequent pairing with the next complementary base in the template (dNTP-stabilized misalignment). B). Synthesis on DNA containing an abasic
site. dNTP-stabilized misalignment is predominantly used by DnaE to extend past an abasic lesion generating a (-1) deletion in the template strand.

Abbreviations used: DTT, dithiotheitol; Pol, DNA polymerase; dNTP, deoxyribonucleoside triphosphates; ATP, adenosine triphosphate; SSB, Single-Strand Binding protein; abasic site, tetrahydrofuran moiety; 2AP, 2-aminopurine; 8-oxo-dG, 8-oxo-7, 8-dihydro-2'-deoxyguanosine; BSA, bovine serum albumin.
| Template : Primer | $V_{MAX}/K_{M}$ | $I_{inc}$ |
|------------------|-----------------|---------|
| A:A              | $6.3 \times 10^{-10}$ | $4.9 \times 10^{-11}$ |
| A:T              | $1.5 \times 10^{-9}$ | 1.0     |
| A:C              | $7.5 \times 10^{-10}$ | $5.8 \times 10^{-11}$ |
| A:G              | $6.8 \times 10^{-10}$ | $5.9 \times 10^{-11}$ |
| T:A              | $1.7 \times 10^{-10}$ | 1.0     |
| T:T              | $4.6 \times 10^{-9}$ | $2.5 \times 10^{-11}$ |
| T:C              | $2.5 \times 10^{-10}$ | $1.5 \times 10^{-11}$ |
| T:G              | $3.8 \times 10^{-10}$ | $1.9 \times 10^{-11}$ |
| C:A              | $6.0 \times 10^{-10}$ | $2.2 \times 10^{-11}$ |
| C:T              | $6.6 \times 10^{-9}$ | $3.7 \times 10^{-11}$ |
| C:C              | $3.0 \times 10^{-10}$ | $1.7 \times 10^{-11}$ |
| C:G              | $1.8 \times 10^{-10}$ | 1.0     |
| G:A              | $3.9 \times 10^{-10}$ | $1.4 \times 10^{-11}$ |
| G:T              | $6.1 \times 10^{-10}$ | $1.5 \times 10^{-11}$ |
| G:C              | $2.8 \times 10^{-10}$ | 1.0     |
| G:G              | $6.1 \times 10^{-10}$ | $1.8 \times 10^{-11}$ |

| 2AP:A            | $6.7 \times 10^{-10}$ | $5.2 \times 10^{-11}$ |
| 2AP:T            | $7.7 \times 10^{-10}$ | $6.0 \times 10^{-11}$ |
| 2AP:C            | $2.3 \times 10^{-10}$ | $1.8 \times 10^{-11}$ |
| 2AP:G            | $2.2 \times 10^{-10}$ | $1.7 \times 10^{-11}$ |
\[ \text{template: } \begin{align*}
32P5' & : \text{GCG} \downarrow \\
3' & : \text{CCGAACATGACAGCAGCAACC} \end{align*} \]

\[ \begin{align*}
\text{primer} \end{align*} \]

A) S. pyogenes Dna E

B) E. coli core(exo-)

C) S. pyogenes PolC

Fig 1
A) Deoxynucleotide incorporation from matched and mismatched primer/template termini by DnaE

B) 

| Template/Primer | $V_{\text{max}}/K_m$ | $\rho_{\text{ext}}$ |
|-----------------|---------------------|-------------------|
| A:A             | $1.8 \times 10^{-08}$ | $6.0 \times 10^{-08}$ |
| A:T             | $3.0 \times 10^{-08}$ | 1.0               |
| A:C             | $3.7 \times 10^{-08}$ | $1.2 \times 10^{-02}$ |
| A:G             | $1.4 \times 10^{-04}$ | $4.8 \times 10^{-04}$ |
| T:A             | $9.0 \times 10^{-01}$ | 1.0               |
| T:T             | $4.7 \times 10^{-04}$ | $5.2 \times 10^{-04}$ |
| T:C             | $4.4 \times 10^{-04}$ | $4.9 \times 10^{-04}$ |
| T:G             | $1.2 \times 10^{-04}$ | $1.3 \times 10^{-04}$ |
| G:A             | $7.4 \times 10^{-08}$ | $1.2 \times 10^{-08}$ |
| G:T             | $1.3 \times 10^{-04}$ | $2.1 \times 10^{-04}$ |
| G:C             | $8.8 \times 10^{-06}$ | $8.4 \times 10^{-06}$ |
| G:G             | $6.4 \times 10^{-03}$ | 1.0               |
| G:A             | $1.4 \times 10^{-08}$ | $3.2 \times 10^{-08}$ |
| G:T             | $2.1 \times 10^{-02}$ | $4.7 \times 10^{-02}$ |
| G:C             | $4.4 \times 10^{-04}$ | 1.0               |
| G:G             | $2.8 \times 10^{-04}$ | $5.3 \times 10^{-04}$ |

C)
### A)

\[ 3^{2}\text{P}5' - \text{GGC} - 3' \]

\[ 3' - \text{CCG}X\text{ACTAATGACGACAGCAACC} \]

- DNAs: dA, dT, dC, dG
- Time points: 0.5, 1.0, 5.0 min

### B)

\[ 3^{2}\text{P}5' - \text{GGC} - 3' \]

\[ 3' - \text{CCG}X\text{ACTAATGACGACAGCAACC} \]

- DNAs: dNTP
- Time points: 0.5, 1.0, 5.0 min

**Sp DnaE**

**Ec core (exo-)**

**Sp PolC**
A) *Sp* DnaE

B) *Ec* core (exo-)

Fig 5
A) Time course of DNA polymerase activity.

B) Gel electrophoresis showing DNA polymerases.

C) DNA sequences and concentrations used in the experiment.
A) 5'-GGCT 3'-CCGGAGTAAI  
\[\text{direct misincorporation}\]  
5'-GGCT 3'-CCGGACTAAI

B) 5'-GGCT 3'-CGGGAGTAAI  
\[\text{dNTP-stabilized misalignment}\]  
5'-GGCT 3'-CCGGACTAAI
The essential C-family DnaE polymerase is error-prone and efficient at lesion bypass
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