DNA exonucleases are critical for DNA replication, repair, and recombination. In the bacterium *Escherichia coli* there are 14 DNA exonucleases including exonuclease I-IX (including the two DNA polymerase I exonucleases), RecJ exonuclease, SbcCD exonuclease, RNase T, and the exonuclease domains of DNA polymerase II and III. Here we report the discovery and characterization of a new *E. coli* exonuclease, exonuclease X. Exonuclease X is a member of a superfamily of proteins that have homology to the 3'-5' exonuclease proofreading subunit (DnaQ) of *E. coli* DNA polymerase III. We have engineered and purified a (His)_6-exonuclease X fusion protein and characterized its activity. Exonuclease X is a potent distributive exonuclease, capable of degrading both single-stranded and duplex DNA with 3'-5' polarity. Its high affinity for single-strand DNA and its rapid catalytic rate are similar to the processive exonucleases RecJ and exonuclease I. Deletion of the exoX gene exacerbated the UV sensitivity of a strain lacking RecJ, exonuclease I, and exonuclease VII. When overexpressed, exonuclease X is capable of substituting for exonuclease I in UV repair. As we have proposed for the other single-strand DNA exonucleases, exonuclease X may facilitate recombinational repair by pre-synaptic and/or post-synaptic DNA degradation.

Examination of multiple sequence alignments by both BLAST (1) and hidden Markov model (2) have helped define a large family of proteins that share sequence homology with the 3'-5' exodeoxyribonuclease domain of DNA polymerases. The ε proofreading subunit of *Escherichia coli* DNA polymerase III, encoded by dnaQ, is the archetypal member of this family. Other family members include the bacterial proteins RNase T, RNase D, exonuclease I (Exol), oligoribonuclease (3), the Saccharomyces cerevisiae PAN2 protein, and the human Werner syndrome protein (WRN) (1, 2, 4). These proteins share a conserved tripartite set of “Exo” motifs containing negatively charged aspartate and glutamate residues (5). These hallmark residues can be visualized in the crystal structure of the Klenow (proofreading) subunit of *E. coli* DNA polymerase I to coordinate two divalent cations that catalyze DNA phosphodiester bond cleavage (6–9). Comparison of the crystal structures of the Klenow fragment and bacteriophage T4 DNA polymerase suggests that the Exo motifs are diagnostic of functional conservation, since both proteins share the same active site structure despite the lack of sequence identity outside of the Exo motifs (2, 10). Presumably, other proteins that share these motifs adopt a catalytic site structure and mechanism of action similar to the polymerase exonuclease domain.

It has recently been demonstrated that the Werner syndrome protein (WRN) has a 3'-5' DNA exonuclease activity (11–13). Originally identified as a 3'-5' RecQ-like DNA helicase (14–16), the Werner syndrome protein also has an N-terminal DnaQ-like nuclease domain (17, 18). WRN possesses a weak exonuclease activity with specificity for the 3'-ending recessed strand of a partial DNA duplex but is unable to degrade single-strand DNA alone (12).

We recently reported that RNase T of *E. coli*, previously described as a 3'-5' ribonuclease (19–21), also possessed a potent 3' to 5' distributive single-strand (ss) DNA-specific exonuclease activity (22). When overexpressed, RNase T was capable of complementing DNA repair defects caused by a deficiency in *E. coli* Exo I. Unlike exonucleasees associated with DNA polymerase, which can degrade from the 3' end of double-strand (ds) DNA molecules, RNase T had no activity on dsDNA substrates (22). Clearly, the architecture of proteins within the DnaQ superfamily allows for different modalities of function, since the same active site configuration can be used to accommodate various substrates (ssDNA, dsDNA, RNA) while retaining the 3'-5' polarity of degradation.

In addition to the bacterial members of the DnaQ superfamily listed above is an open reading frame of unknown function designated *yobC* (also known as O220 and b1844) at 41.5 min on the *E. coli* chromosome. We have cloned, overexpressed, and characterized the protein product of this gene. Overexpression of this gene concomitantly induces high levels of a Dnase activity on both ssDNA and dsDNA. We have renamed this open reading frame *exoX* and the native 25-kDa protein product exonuclease X (*ExoX*). We have purified a (His)_6-*ExoX* fusion protein to homogeneity and characterized its nuclease activity on various DNA substrates. *ExoX* is an extremely potent 3' to 5' distributive nuclelease capable of degrading 40-kilobase bacteriophage T7 ssDNA and dsDNA to completion. Its affinity for ssDNA ends is greater than for dsDNA, and it appears to have no affinity for RNA. When overexpressed, *ExoX*, like RNase T (23), is capable of substituting for Exol in *vivo*, as measured by its ability to increase the UV survival of an Exol-deficient strain. A mutation in *exoX* did not by itself cause sensitivity to UV but strongly augmented the UV sensitivity of a strain deficient in ssDNA exonucleases RecJ, Exol, and ExoVII.

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‡ The abbreviations used are: ExoI, exonuclease I; ExoVII, exonuclease V; *ExoX*, exonuclease X; ss, single-strand; ds, double strand; DTT, dithiothreitol; BSA, bovine serum albumin; PCR, polymerase chain reaction; kb, kilobase(s).
EXPERIMENTAL PROCEDURES

Plasmid Constructions

The exoX gene was amplified by PCR using Pfu polymerase (Stratagene Inc.) from wild type *E. coli* strain MG1655 genomic DNA using primers 5'-CGGATTCTTACAGGACTGATCCTGAGAATC-3' and 5'-GGTCTAGACTGATAAGTTTCCG-3' and buffer conditions recommended by the manufacturer. Primers were annealed to the genomic DNA at 50 °C for 30 s and extended for 2 min at 72 °C; 25 cycles of PCR were performed. The PCR product was subsequently digested with restriction endonucleases EcoRI and XbaI and ligated into the compatible sites of pBSSK (Stratagene Inc.), producing pEXO. Sequence analysis verified the construct was error-free.

The EcoRI-XbaI fragment from pEXO was cloned into compatible sites within pBSKS-S (Stratagene Inc.) creating pEXOXS. Plasmid pEXOXS, a derivative of pEXOXS, with a frameshift mutation 171 bases pairing downstream from the initiation codon of *exoX*, was constructed by cleavage of pEXOXS DNA with restriction endonuclease *NcoI*, "fill-in" synthesis with Klenow fragment (DNA polymerase I), and blunt end DNA ligation. An *exoX* (His)_6-tagged gene fragment was constructed by cloning the 693-base pair BamHI-SmaI fragment of pEXO into the same sites within pET28a (+) (Novagen), producing the plasmid pEXO-His.

A 2.919-base pair region of the *E. coli* chromosome containing the *exoX* gene was amplified by PCR using primers beginning 1,088 base pairs upstream (5'-GGGATCCGGATCCATTGTCATGTG-3') and 1,167 base pairs downstream (5'-GGTCTAGAGCATGATCCTGAG-3') of *exoX*. The PCR product was performed using Turbo Pfu polymerase (Stratagene Inc.) in buffer conditions recommended by the manufacturer. Primers were annealed to MG1655 *E. coli* genomic DNA at 60 °C for 30 s and extended for 3 min at 72 °C; 25 cycles of PCR were performed. The PCR product was digested with XbaI and EcoRI and ligated into compatible sites within the Litmus 29 vector (New England Biolabs Inc.), creating the plasmid pEXOXFlank.

A precise deletion of the *exoX* open reading frame from the plasmid pEXOXFlank was performed by PCR. The primers utilized for the PCR flanking *exoX* were designed and were oriented to replace the entire plasmid except for the *exoX* open reading frame. Both primers: 5'-GGGATCCGGATCCGGATCCATTGTCATGTG-3' and 5'-GGTCTAGAGCATGATCCTGAG-3' contained NotI sites at the primer 5' terminus. The PCR was performed as above except that primer annealing was at 45 °C, and extensions were performed for 6 min. The resulting 5-kb PCR product was treated with DpnI to remove any original methylation template DNA and then digested with NotI and ligated to a 2.1-kb NotI fragment from plasmid pMK155 (24) containing a Tn5 npt gene, which confers kanamycin resistance. Flanking *npt* on both sides are 140-base pair restriction sites from the broad host range plasmid RP4 multimer donor for further strain constructions by P1 transduction (Ref. 30 and see below). With the exception of STL2329 and STL4525, all strains designated STL were derived from BT199 and *lin*-sensitive isolates were analyzed by Southern blot (29) and were verified by restriction analysis.

**Strains**

For Protein Expression—STL2350 (sonA2 recC284-7:Gm ζxseA-guaB) zff-3139:TN10kan (ΔlacI31lacZΔM15) was used for Eox protein expression. Plasmid pTH30 (25), carrying a heat shock-inducible T7 RNA polymerase, was introduced into STL2350 by transformation (26) and selection for chloramphenicol resistance. For protein-labeling experiments pET28a and pBSSK were transformed into STL2350/pTJH30 cells selecting ampicillin resistance. Flanking *npt* on both sides are 140-base pair restriction sites from the broad host range plasmid RP4 multimer donor for further strain constructions by P1 transduction (Ref. 30 and see below). With the exception of STL2329 and STL4525, all strains designated STL were derived from BT199 and *lin*-sensitive isolates were analyzed by Southern blot (29) and were verified by restriction analysis.

**Protein Expression and Analysis**

Eox protein expression was induced from the T7 80 promoter of pEXO by 42 °C heat shock induction of the T7 RNA polymerase gene on plasmid pTH30. T7 promoter-mediated expression of the (His)_6-Eox fusion protein from plasmid pEXO-His was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (1 mM) to ST4337 (pEXO-His) containing 25 mM sodium Iodide and 25 mM L-threonine. Kanamycin-resistant and *ampC*-sensitive isolates were analyzed by Southern blot (29) and were found to have the appropriate deletion/insertion at the *exoX* locus. One such isolate, STL4525 (ΔexoX1::npt recC284-7:Gm ζxseA-guaB) zff-3139:TN10kan Δ LacI31lacZΔM15) was used as a donor for further strain constructions by P1vir-mediated transduction (Ref. 30 and see below). With the exception of STL2352 and STL4525, all strains designated STL were derived from BT199 and carry additional genetic markers (F _Δ_ thi-1 Δgpt-proA82 thr-1 leuB6 hsdR17 rfdB1 ara-14 lacI1 galK2 xyl-5 mit-1 t3x-33 supE44 rpsL31 recA ).

**UV Survival Assays**—pEXOXS, pEXOXS, and pBSKS were transformed (26) into either STL2701 (ΔsonA300::cat ζxseA-guaB) zff-3139:TN10kan rec2052::TN10kan) or STL2348 (ΔxseA-guaB) zff-3139:TN10kan rec3284:TN10, selecting ampicillin. In addition, various isogenic exonuclease-deficient mutants in the BT199 genetic background (listed in Fig. 7) were assayed for UV survival. The mutant alleles used in the construction of the various exonuclease-deficient strains were: _ΔexoX1::npt_, for Eox; STD4330:cat, for ExoI; rec3284:TN10 for RecJ; and xseA18::amp for ExoVII. Details of the construction of these strains will be published elsewhere and are available by request from the authors.

**Protein Expression and Analysis**

Eox protein expression was induced from the T7 80 promoter of pEXO by 42 °C heat shock induction of the T7 RNA polymerase gene on plasmid pTH30. T7 promoter-mediated expression of the (His)_6-Eox fusion protein from plasmid pEXO-His was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (1 mM) to ST4337 (pEXO-His) containing 25 mM sodium Iodide and 25 mM L-threonine. Kanamycin-resistant and *ampC*-sensitive isolates were analyzed by Southern blot (29) and were found to have the appropriate deletion/insertion at the *exoX* locus. One such isolate, STL4525 (ΔexoX1::npt recC284-7:Gm ζxseA-guaB) zff-3139:TN10kan Δ LacI31lacZΔM15) was used as a donor for further strain constructions by P1vir-mediated transduction (Ref. 30 and see below). With the exception of STL2352 and STL4525, all strains designated STL were derived from BT199 and carry additional genetic markers (F _Δ_ thi-1 Δgpt-proA82 thr-1 leuB6 hsdR17 rfdB1 ara-14 lacI1 galK2 xyl-5 mit-1 t3x-33 supE44 rpsL31 recA ).
mm NaCl, 25 mm imidazole, and 1 mm β-mercaptoethanol. Buffer C contained 10% glycerol, 20 mm Tris-Cl (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol (DTT). STLA533 (pEXO-His transformant of BL21) was cultured at 37 °C in 34 liters of LB + Km to an A600 of 0.6, then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h. Cells were harvested and frozen as described previously (33) in a volume of 700 ml. A crude extract was prepared by lysing cells in 10% sucrose, 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mM EDTA, and 1 mg/ml lysozyme for 1 h on ice. Three cycles of freeze/thawing (10 min 37 °C, 10 min 0 °C) were performed before a supernatant was obtained by high speed centrifugation at 130,000 × g for 20 min. A crude extract (700 ml, 1.8 g of protein per ml) was adjusted to 200 mM NaCl and 5 mM imidazole before adding 15 ml of Ni2+-nitrilotriacetic acid-garosar resin (Qiagen) equilibrated in Buffer A. The resin and extract slurry was allowed to mix for 8 h with stirring at 4 °C. The resin was washed by repeated low speed centrifugation and resuspension in 175 ml of Buffer A, 85 ml of Buffer B, and finally with 50 ml of Buffer C + 200 mM NaCl. Proteins were eluted from the resin with 35 ml of Buffer C + 100 mM NaCl + 400 mM imidazole. The resulting fraction (6.0 mg of protein) was concentrated to 10 ml using a Centriprep 10 (Amicon) cartridge and then dialyzed against 2 l changes of Buffer A. This fraction was then applied to a 0.5-m1 dsDNA cellulose column equilibrated in Buffer C + 25 mM NaCl and washed with 10 ml of Buffer C. Bound proteins were eluted in a single step to Buffer C + 500 mM NaCl containing nuclease activity were pooled and dialyzed overnight against 0.5 liters of 60% glycerol, 500 mM NaCl, and 1 mM EDTA, and then again against 60% glycerol, 1 mM EDTA. The purified protein (0.75 mg at 0.41 mg/ml) was stored at −20 °C.

**DNA Substrates and DNase Assays**

Uniformly labeled bacteriophage T7 [3H]DNA with a specific activity of 2.5 × 104 cpm/nmol of nucleotide was prepared as described previously (33) using [3H]thymidine (NEN Life Science Products). 3' end-labeled substrate was generated by Klenow fragment fill-in synthesis of HindIII-digested pBSSK DNA, with [γ-32P]ATP. 5' end-labeled substrate was generated from HindIII-digested pBSSK DNA, treated with shrimp alkaline phosphatase, and phosphorylated with T4 polynucleotide kinase and [γ-32P]ATP. Both 3' and 5' end-labeled substrates were purified by G-50 Sephadex quick spin column (Roche Molecular Biochemicals). Unless otherwise stated, enzyme assays employed 0.5 μg of T7 [3H]DNA (1.5 nmol) or 0.5 μg (0.5 pmol of ends) of 3' or 5' end-labeled substrate. For ssDNA assays, substrates were incubated for 5 min at 100 °C then quenched on ice. Standard reactions contained 10 mM Tris-Cl (pH 8.0), 10 mM MgCl2, 1 mM NaCl, and 2 mg/ml BSA in 50 μl (for T7 substrates) or 30 μl (for end-labeled substrates). Competition assays were performed with 50 ng (50 fmol) of 3' end-labeled substrate, varying the amount of either single-strand or double-strand cold competitor DNA. Competition reaction was utilized E. coli RNA trix (Sigma) and Torula yeast RNA (molecular mass range 3 × 104–4 × 108 g/mol, Sigma). Both RNAs are used as Sigma as ribonuclease assay substrates. Cold RNA stocks were prepared before each competition experiment in cold sterilizer water. The A260 of the RNA stocks was determined immediately before use to verify the optical density provided by the manufacturer and to ensure that no degradation had occurred. Assays were performed by incubating reaction containing the lower molecular weight band is not known; however, it may be a proteolytic product of the (His)6-ExoX protein, since it bound to the dsDNA cellulose affinity column. Furthermore, the protein that bound to the dsDNA cellulose column had significant nuclease activity, whereas the flow-through fraction from the dsDNA cellulose column containing the lower molecular weight protein had

**RESULTS**

**Overexpression and Purification of a DNase Activity Associated with Exonuclease X**—The E. coli open reading frame designated yobC, hereafter known as exoX, was amplified by PCR and cloned directionally into pBSKK−, placing its expression under T7 promoter control. Induction of exoX expression from the T7 promoter of pEXO-His to the production of a single 25-kDa protein, consistent with the expected molecular mass of the protein (Fig. 1A, lane 4). The 25-kDa protein was absent in uninduced extracts (Fig. 1A, lane 3) and from cells carrying vector only (Fig. 1A, lane 2). Crude extracts prepared from these induced cells were tested for DNase activity using uniformly [3H]-labeled T7 DNA substrates. Strains induced for expression of exoX exhibited a 150-fold increase in ssDNA activity and a 280-fold increase in dsDNA activity compared with cells carrying vector alone (Table I). Both ssDNAse and dsDNAse activities were Mg2+-dependent, as no increase in activity was noted in the absence of the divalent cation (data not shown).

An N-terminal (His)6-tagged ExoX protein fusion was constructed using the pET28a (+) vector (Novogen). (His)6-ExoX protein was expressed from the T7 promoter on plasmid pEXO-His (Fig. 1A). Crude extracts prepared from induced cells carrying pEXO-His were tested for DNase activity. Strains induced for expression of (His)6-ExoX exhibited a 47-fold increase in ssDNA activity and a 170-fold increase in dsDNA activity compared with cells carrying vector alone (Table I). Comparison of uninduced levels of expression between the pET28a (+) vector and pEXO-His revealed a basal level of expression of the (His)6 fusion protein without induction of the T7 promoter. Upon induction, increased expression of two [35S]methionine-labeled proteins was noted. The identity of the second band is unknown; however, it did not appear (Fig. 1A, lane 4) when the native protein was expressed in a different strain background.

The (His)6-ExoX protein was overexpressed in E. coli strain BL21 and was purified using nickel-agarose chromatography (Fig. 1B). Three high molecular weight contaminants and a lower molecular weight protein were removed by dsDNA cellulose affinity chromatography. The identity of the abundant lower molecular weight band is not known; however, it may be a proteolytic product of the (His)6-ExoX protein, since it bound to the Ni2+-nitrilotriacetic acid resin yet failed to bind dsDNA cellulose column. Furthermore, the protein that bound to the dsDNA cellulose column had significant nuclease activity, whereas the flow-through fraction from the dsDNA cellulose column containing the lower molecular weight protein had

| Plasmid | ssDNA Specific activity | dsDNA Specific activity |
|---------|-------------------------|-------------------------|
| pBSSK−  | 50                      | 10                      |
| pEXO-His| 7600                    | 2800                    |
| pET28a (+) | 110                  | 20                      |
| pEXO-His | 5200                    | 3400                    |

**Enzymes and Antibiotics**

Shrimp alkaline phosphatase was obtained from Amersham Pharmacia Biotech. Lysozyme was obtained from U. S. Biochemical Corp. All other enzymes were obtained from New England Biols, Inc. The antibiotics ampicillin, kanamycin, and chloramphenicol were used at 100, 60, and 15 μg/ml, respectively.
nearly none. The purified protein was analyzed by mass spectrometry and was determined to have a molecular mass of 28,552 mass units. The molecular mass determined by mass spectrometry is commensurate with the expected mass of the fusion protein by amino acid sequence.

Properties of the DNase Activity of Exonuclease X—Purified (His)_6-ExoX was used to determine optimal conditions for ssDNase activity. ExoX showed optimal activity at pH 8.0 in the presence of Mg^{2+} (Fig. 2A). Similar to other nucleases, the ssDNase activity of ExoX was dependent upon the presence of the divalent cation Mg^{2+}; no detectable degradation was seen in its absence or in the presence of Mn^{2+} (Fig. 2B). BSA enhanced the ssDNase activity of ExoX (Fig. 2C). The ssDNase activity of ExoX was enhanced with low salt concentrations, but above 5 mM salt, the activity decreased with increasing concentration (Fig. 2D). The addition of the sulfhydryl reducing agent, DTT (1–5 mM), did not alter levels of ssDNase activity (data not shown).

In reactions with denatured bacteriophage T7 DNA (40 kb in length), 1 ng (34 fmol) of purified (His)_6-ExoX linearly degraded 35% of the total DNA (0.5 μg, 1.5 nmol in nucleotides) in 20 min. The addition of 10 ng of protein resulted in 100% of the ssDNA substrate being degraded (Fig. 3A). Similarly, in reactions with T7 dsDNA, 5 ng (0.17 pmol) of purified (His)_6-ExoX linearly degraded 30% of the total DNA in 20 min, and the addition of 40 ng (1.4 pmol) of (His)_6-ExoX resulted in 100% of the dsDNA substrate being degraded (Fig. 3B). Using data points in the linear range of T7 DNA degradation from Fig. 3, the calculated rate of nucleotide release/protein monomer is 800 nucleotide/min for ssDNA, and 150 nucleotides/min for dsDNA. From its ability to degrade T7 DNA completely, we conclude that ExoX has little or no specificity for DNA sequence or structure. No endonuclease activity was observed in reactions with ΦX174 circular ssDNA, supercoiled dsDNA, or relaxed dsDNA, suggesting that the DNase activity of ExoX is exo-3′-5′-exonuclease and not endonucleolytic in nature (data not shown). Electrophoretic analysis of end-labeled reaction products (discussed below) also support this conclusion.

Using uniformly labeled T7 ssDNA as substrate, the extent of ssDNA degradation was determined for a substrate concentration range of 0.2–3.0 nM with 80 ng/ml (2.8 nmol monomer) of (His)_6-ExoX in 5-min reactions using standard buffer conditions. A Lineweaver-Burk plot produced a K_m of 1.7 mM and a V_max of 50 nmol of nucleotide/min/mg of protein for the degradation of 40 kb of ssDNA molecules (data not shown). The K_cat at high T7 ssDNA substrate concentrations (based on V_max) is 1,400 nucleotides/min/monomer.

The DNase activity of (His)_6-ExoX was examined on various end-labeled ssDNA and dsDNA substrates (Fig. 4). Purified (His)_6-ExoX showed efficient release of the terminal nucleotide from 3′ ssDNA ends. 95% of all 3′ termini were removed in 20 min with a 1:1,900 ratio (protein monomer to DNA, Fig. 4A). Release of the terminal nucleotide from 5′ ssDNA ends was less efficient; nearly 500-fold more enzyme was required to achieve comparable extent of release of 5′ ssDNA ends (Fig. 4B). In reactions with dsDNA, complete release of the terminal nucleotide from 3′ dsDNA ends (5′ overhangs) required approximately 10-fold more enzyme (Fig. 4C) as compared with similarly end-labeled ssDNA (Fig. 4A). Nucleolytic activity was barely detected with 5′-labeled dsDNA substrate (Fig. 4D). These results imply that ExoX acts as a 3′ to 5′ exonuclease on both ssDNA and dsDNA substrates, with ssDNA more efficiently attacked than dsDNA. A 3′ to 5′ polarity of digestion was confirmed by gel electrophoresis of ExoX nuclease reactions with end-labeled ssDNA and dsDNA as substrate. In these reactions BSA was omitted to allow DNA electrophoresis without manipulation of the samples after the reaction; subsequently more enzyme (2–3-fold) was needed for the reactions to go to completion as compared...
with the values seen in Fig. 2. A 1:700 molar ratio of (His)$_6$-ExoX protein to 3'-end labeled ssDNA (protein monomers: 3’ DNA ends) produced a loss of the terminal 3' label from nearly all the substrate DNA molecules by 20 min, without detectable shortening of the labeled DNA (Fig. 5A). In contrast, incubation of (His)$_6$-ExoX with 5' end-labeled ssDNA at a molar ratio of 1:5 (protein monomers: 5’ DNA ends) resulted in progressive shortening of the labeled DNA with little loss of signal intensity at the earlier time points (Fig. 5B). By 10 min the ssDNA molecules either were degraded completely or had become heterogeneous in size due to asynchronous digestion (Fig. 5B). A 1:100 ratio of (His)$_6$-ExoX protein to 3’ end-labeled dsDNA (protein monomers: 3’ DNA ends) also produced a loss of the terminal 3’ label from nearly all the substrate dsDNA molecules by 20 min without detectable shortening of the labeled DNA (Fig. 5C). Reaction of a 3-kb 5’ end-labeled dsDNA substrate with (His)$_6$-ExoX in a 1:2 ratio (protein monomers: 5’ DNA ends) resulted in progressive shortening of the dsDNA molecules from the 3’ ends; eventually shortening from both ends resulted in a 1.5-kb ssDNA product, seen at 10 min.

These results are consistent with a 3' to 5' polarity of DNA degradation by ExoX. Furthermore, the degradation by ExoX must be via a distributive mechanism, because the substrate molecules appear to be degraded uniformly from the 3' end when substrate is in excess of enzyme. ExoX must dissociate and rebind to its ssDNA substrate during cycles of degradation, similar to the mechanism found for the ssDNA nuclease activity associated with RNase T of E. coli and in contrast to the processive mechanism of DNA degradation exhibited by ssDNA exonucleases such as exonuclease I (35) and RecJ$^2$.

Competition experiments were performed using 3’ end-labeled ssDNA as the assay substrate and either ssDNA, dsDNA, tRNA, or yeast RNA as unlabeled competitors. ssDNA proved to be a potent competitor with a 1000-fold excess of cold ssDNA, producing an 80% decrease in released counts. At the same ratio of substrate to competitor, dsDNA showed a modest 6% decrease in counts released, whereas both RNA species failed to compete altogether. These results demonstrate that the affinity of ExoX for ssDNA is considerably greater than for dsDNA and that RNA is not a substrate.

ExoX Is Involved in the Repair of UV-induced DNA Damage—To determine whether the 3'-5' nuclease activity of ExoX could serve a biological function we asked whether high copy expression of ExoX could substitute for ExoI deficiency in UV repair. Plasmid pEExoXKS$^+$ with exoX under lac promoter control was transformed into both RecJ$^+$ ExoI$^-$ ExoVII$^-$ and RecJ$^+$ ExoI$^+$ ExoVII$^+$ strains and assayed for UV survival. A plasmid containing exoX was capable of ameliorating the UV repair defect of a RecJ$^+$ ExoI$^+$ ExoVII$^+$ strain, however it had no effect upon the UV sensitivity of a RecJ$^+$ ExoVII$^-$ strain (Fig. 6). Neither vector (pBSKS$^+$) nor plasmid pEExoXSs, containing a frameshift early in the exoX coding region, provided any measure of protection. These results demonstrate that ExoX can specifically compensate for loss of ExoI, a 3’-5’-specific exonuclease, in vivo.

$^2$ S. T. Lovett and R. D. Kolodner, unpublished observation.
To confirm a role for ExoX in DNA repair, a null mutant of ExoX was constructed by homologous recombination of plasmid pExoXΔ into the E. coli chromosome. This null mutant, ΔexoX::npt, carries a precise deletion of the exoX open reading frame replaced by a cassette containing the npt gene conferring kanamycin resistance. Using this null allele of exoX, a series of isogenic exonuclease-deficient strains were constructed. The exoX null mutation was added to strains already deficient in one or more of the other known single-strand exonucleases; RecJ, ExoI, and ExoVII. We assayed this set of strains for their ability to survive UV irradiation (Fig. 7). An exoX null mutant alone had no measurable effect upon UV survival compared with a Exo+ strain. Similarly, the ΔexoX::npt allele added to any other single or double exonuclease mutant had no effect on UV survival compared with their respective progenitor strain. However, the ΔexoX::npt null allele added to a RecJ− ExoI− ExoVII− mutant resulted in a strain that was significantly more UV sensitive than the triple mutant alone, demonstrating a synergistic relationship among these nucleases with respect to UV survival.

Fig. 6. Complementation of ExoI deficiency by ExoX. STL2701 RecJ− ExoI− ExoVII− (filled symbols) or STL2348 RecJ− ExoVII− (open symbols) were transformed with either pExoXKS− (●, □), pExoXfs (●, ○), or pBSKS− (△, △). Transformants were assayed for survival after UV irradiation as described previously in Viswanathan et al. (23).

**DISCUSSION**

In E. coli, DNA exonucleases play diverse and important roles in DNA metabolism. The 3'-5' exonuclease associated with the three polymerases (dnaQ, polA, polB) help maintain genomic fidelity during replication. Exonuclease V, better known as the RecBCD nuclease, is important for conjugal and repair recombination of double-strand breaks (36). Exonuclease VIII, the product of the cryptic Rac prophage, is a component of the RecE pathway of recombination in E. coli (37). There are also three known processive ssDNA-specific exonucleases in E. coli, exonuclease I, exonuclease VII, and RecJ exonuclease (38). These exonucleases catalyze the nucleolytic cleavage of successive phosphodiester bonds on a ssDNA molecule. Exonuclease I degrades ssDNA in a 3' to 5' direction (38), RecJ exonuclease has 5' to 3' polarity (33), and exonuclease VII possesses dual polarity acting from either DNA end (39). Mutation of one or several of these genes in combination has pleiotropic effects in DNA repair and recombination in E. coli, including sensitivity to UV irradiation and recombination defects (34, 40, 41). All three of these exonucleases have been additionally implicated in the process of methyl-directed mismatch repair (42, 43). Using in vitro reconstitution experiments with purified proteins, all three exonucleases can mediate the excision step of mismatch repair using synthetic mismatch repair substrates (42). In vivo experiments with strains multiply deficient in all three exonucleases have, however, failed to demonstrate a role for these exonucleases in mismatch repair, suggesting that other, unknown exonucleases exist in E. coli capable of compensating for the loss of ExoI, ExoVII, and RecJ (34, 40).

We have demonstrated that the E. coli open reading frame yobC, which has homology to the DnaQ superfamily, encodes a 25-kDa protein that possesses a potent Mg2+-dependent ssDNA exonuclease activity. Accordingly, we have renamed this open reading frame exoX and its protein product Exonuclease X. The exoX gene appears to be part of a cisron with another unknown open reading frame denoted yobB. The genes are separated by 23 nucleotides. Although exoX does not appear to have any promotor of its own, the region upstream of yobB contains a σ70 promoter-like sequence. Bacterial genome data base searches revealed that both exoX and yobB have protein homologs in Salmonella typhimurium; in addition, yobB has a second
3'-5' Single-strand DNase Activity of E. coli Exonuclease X

homolog in Pseudomonas aeruginosa. No other strongly homologous proteins were found in the eubacterial data bases, including the proteobacterial genomes of Haemophilus influenzae and Helicobacter pylori. ExoX did, however, have significant homology with the DNA polymerase exonuclease domain of Bacillus subtilis and lesser homology to the DNA polymerase ε subunits of various eubacterial species including Chlamydia trachomatis and Staphylococcus aureus.

Here we report the purification of a (His)_6-ExoX fusion protein and the characterization of its nuclease activity on various DNA substrates. ExoX degrades DNA in a 3' to 5' direction using a distributive mechanism of hydrolysis. Therefore, unlike the processive nucleases ExoI, ExoX undergoes multiple rounds of binding, hydrolysis, and release to degrade long substrate molecules. Its rapid rate of ssDNA degradation (1,400 nucleotides ssDNA/min/monomer) is due in part to the high affinity of the enzyme (K_m = 1.7 nM) for ssDNA. Although capable of degrading both ssDNA and dsDNA molecules, ExoX has higher affinity for ssDNA ends as judged by the extent of degradation of various substrates and by competition experiments. The need for high enzyme to DNA stoichiometry for duplex DNA degradation may mean that the duplex DNA binding step is slow relative to ssDNA binding. The mechanism by which ExoX binds and catalyzes phosphodiester bond cleavage of either the single-strand or duplex DNA end may be revealed by more biochemical and structural information.

Previously we demonstrated that RecJ exonuclease, ExoI, and ExoVII are involved in a redundant fashion in the repair of UV-induced lesions (34). We have proposed that these exonucleases act during recombinational repair of lesions that block replication. Here we have demonstrated that ExoX can specifically compensate for UV repair defects associated with the loss of ExoI, itself a potent 3'-5' exonuclease. This result correlates well with the biochemical characterization of the enzyme. The fact that ExoX could only partially compensate for ExoI even when expressed in high copy may reflect the disparity between a processive nuclease such as ExoI and a distributive nuclease like ExoX.

ExoVII, and ExoX is essential for UV repair and potentially other cellular processes.

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