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# Molecular interactions of *Escherichia coli* ExoIX and identification of its associated 3′–5′ exonuclease activity

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## ABSTRACT

**The flap endonucleases (FENs) participate in a wide range of processes involving the structure-specific cleavage of branched nucleic acids. They are also able to hydrolyse DNA and RNA substrates from the 5′-end, liberating mono-, di- and polynucleotides terminating with a 5′ phosphate. Exonuclease IX is a paralogue of the small fragment of *Escherichia coli* DNA polymerase I, a FEN with which it shares 66% similarity. Here we show that both glutathione-*S*-transferase-tagged and native recombinant ExoIX are able to interact with the *E. coli* single-stranded DNA binding protein, SSB. Immobilized ExoIX was able to recover SSB from *E. coli* lysates both in the presence and absence of DNA. *In vitro* cross-linking studies carried out in the absence of DNA showed that the SSB tetramer appears to bind up to two molecules of ExoIX. Furthermore, we found that a 3′–5′ exodeoxyribonuclease activity previously associated with ExoIX can be separated from it by extensive liquid chromatography. The associated 3′–5′ exodeoxyribonuclease activity was excised from a 2D gel and identified as exonuclease III using matrix-assisted laser-desorption ionization mass spectrometry.**

## INTRODUCTION

The flap endonucleases (FENs) are a family of structure-specific nucleases capable of cleaving a variety of branched DNA structures (1,2). These enzymes recognize substrates containing at least one duplex and a single-stranded 5′ overhang such as flaps and pseudo Y structures. The major activity involves cleavage of the phosphodiester backbone one nucleotide into the double-stranded region at the junction of single-stranded bifurcation with duplex DNA (3,4). They also display 5′–3′ exonucleolytic activities on substrates with free 5′-ends (5) and can attack gaps in duplex DNA and covalently closed-circular DNA (6,7). These latter activities appear to be very similar to the gap endonuclease activities described recently in human FEN1 (8). A functional FEN seems to be essential for all organisms. For example, the FEN activity present within the *Streptococcus pneumoniae* DNA polymerase I N-terminal region is essential for cell viability (9), haploinsufficient mice rapidly develop tumours while $FEN^{-}/FEN^{-}$ mutants show embryonic lethality (10).

The striking similarity of the structures of FEN enzymes from a diverse range of organisms has been revealed through crystallographic structure determination. For example, *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Pyrococcus furiosus*, T5 exonuclease (T5FEN), *Thermus aquaticus* DNA polymerase I FEN domain and human FEN1 enzymes share a central beta sheet feature adorned by a number of helices and interconnecting loops (11–16). All possess binding sites for two divalent metal ions composed of conserved glutamyl and aspartyl residues and a DNA-binding feature known as a helix-three-turn-helix motif (17). Based on the observation of two manganese binding sites in *Taq* Pol, Steitz and co-workers (15) proposed a two-metal-ion mechanism for FEN-mediated phosphate ester cleavage similar to that of the Klenow 3′–5′ proof-reading exonuclease (18). The roles of the divalent metal ions include substrate binding by metal ions I and II (19) with the site I metal generating a hydroxide ion essential for catalysis (20). It also appears that just one metal binding site is required for the structure-specific endonuclease activity of the T5FEN enzyme, whereas it requires both metal binding sites to be occupied for exonucleolytic cleavage (19).

The DNA polymerase I protein present in eubacteria possesses FEN activity carried within the N-terminal domain (1). Two groups independently reported the identification of a previously uncharacterized *Escherichia*

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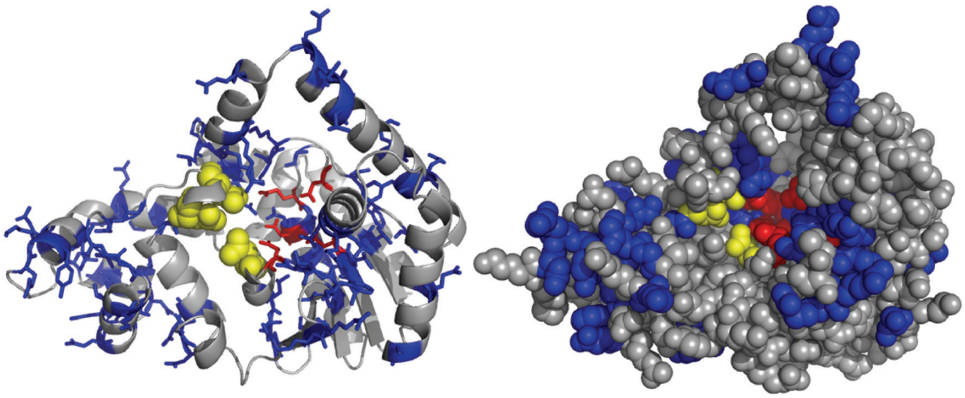

**Figure 1.** Structure of T5FEN showing residues in common with ExoIX. The residues conserved in ExoIX and T5FEN are shown coloured in red or blue are mapped onto the T5FEN crystal structure (pdb code 1exn). Residues in red are the ligands forming the binding site for metal ion I (site I ligands) and are common to both proteins. Left hand panel shows a ribbon representation of the T5FEN backbone and the three metal site II ligands (yellow spacefill) that are absent in ExoIX. In T5FEN, these are aspartates (155, 201 and 204) whereas in ExoIX they are glycine 130, isoleucine 175 and serine 178. A space-filled representation is shown on the right. The figure was generated using MacPyMOL software (http://www.delanoscientific.com).

*coli* DNA polymerase I (Pol I) paralogue by interrogation of sequences deposited in the public databases (21,22). The putative gene, originally designated *exo*, was identified based upon up to 60% sequence identity with part of the 5′ region of *polA*, the gene encoding Pol I. The *exo* gene appeared to have a suitable ribosomal binding site with a GUG start codon but the open reading frame consisted only of 753 nucleotides, with no homology to *polA* detectable beyond this region (21). In comparison, *polA* encodes a much larger protein, consisting of the FEN domain or 5′–3′ exonuclease domain also known as the small fragment and the larger, Klenow fragment carrying the 3′–5′ proofreading and DNA-dependent DNA polymerase activity (5,23). Overall, the 251 amino-acid protein encoded by the *exo* open reading frame displayed 66% similarity with the *E. coli* DNA Pol I protein and most importantly, it appeared to contain most of the residues characteristic of the FENs (24). Interestingly, these include conserved acidic residues involved in binding to one of the essential divalent metal ions usually required for catalysis (site I) but lacks three acidic residues corresponding to site II in T5FEN. Figure 1 shows the crystal structure of the T5FEN with the residues in common with ExoIX identified.

The *exo* gene was renamed *xni* and preliminary characterization of the gene product, expressed as a GST-fusion protein has been reported. A 3′–5′ exodeoxyribonuclease activity was reported for the *xni*-encoded protein and it was renamed exonuclease IX (ExoIX) (25). In addition to this principal exodeoxyribonucleolytic function the protein possessed a deoxyribophosphodiesterase (dRPase) activity on abasic sites (25) and was able to remove 3′ phosphoglycolate end groups from DNA (26). These activities are difficult to reconcile with the strong sequence homology ExoIX shares with the FEN family of nucleases, since they are structurally dissimilar from the apurinic/pyrimidinic endonucleases, which generally perform these types of reactions (27). We were intrigued by the reported 3′–5′ exonucleolytic activity of ExoIX as it appeared to display the reverse of the normal polarity of action seen in the FENs i.e. 5′–3′. In the work reported here, we over-produced native ExoIX and found that by using both one and two-dimensional activity gels, we were able to monitor the protein and nuclease activity during fractionation. Contrary to earlier work (25), the ExoIX protein could be separated from a contaminating 3′–5′ exonuclease activity. We were able to identify the associated 3′–5′ exonuclease activity as being due to low levels of initially co-purifying exonuclease III (ExoIII). Once free of contaminating ExoIII, the ExoIX protein displayed no apparent exodeoxyribonuclease activity under conditions typical for FENs. We also investigated whether ExoIX was able to interact directly with ExoIII or any other proteins present in *E. coli* cell-free extracts. Amongst others, ExoIX bound to single-stranded DNA-binding protein (SSB). The interaction was confirmed using *in vitro* chemical cross-linking. The interaction took place even in the absence of nucleic acids. The SSB tetramer was able to bind up to two monomers of ExoIX.

## MATERIALS AND METHODS

### Cloning and expression of the native *exo*/*xni* gene

The *xni* gene was amplified by PCR from *E. coli* XL-1Blue genomic DNA (Stratagene) using standard procedures (28) with *Taq* polymerase (Promega). The forward primer d(TGAATTCTTTAAGGAGATTATAATGGGCTGTTCA) included the underlined ATG start codon, ribosomal binding site and an EcoRI recognition sequence. The reverse primer d(TAGGGATCCGGCTCGCCGTTA) was designed to incorporate a BamHI recognition site downstream of the *xni* stop codon. After amplification, the PCR product was treated with the above restriction enzymes and ligated into similarly treated expression vector pJONEX4 (6), transformed into *E. coli* BL21(pcI857) and transformants were selected on LB agar plates containing ampicillin at 100 μg ml$^{-1}$. Dideoxy sequencing was used to identify a clone, designated

pJONEX/*xni*, possessing the expected sequence (29). A culture derived from a single colony of *E. coli* BL21 (pJONEX/*xni* pcI857) was grown in 20 l 2× YT (16 g tryptone, 10 g yeast extract, 5 g NaCl/l) containing 25 μg ml$^{-1}$ kanomycin, 100 μg ml$^{-1}$ ampicillin and 100 μg ml$^{-1}$ carbenicillin in a New Brunswick Scientific BIOFLO 4500 Fermenter/Bioreactor, incubated at 30°C, until it reached an $A_{600\,nm}$ of 1.0, at which point the temperature was raised to 42°C for 1.5 h. The vessel was then allowed to cool passively to RT, and incubated overnight. Cells were harvested by centrifugation and washed with 0.9% w/v NaCl, aliquoted into 10–15 g portions and stored at –80°C.

### Purification of ExoIX

Cell-free extracts were prepared free of nucleic acids essentially as described previously from ~10 g cell pellets and stored as an ammonium sulphate slurry (30). The slurry was re-suspended in 50 ml ice-cold 250 mM $KH_2PO_4/K_2HPO_4$, pH 6.5, containing 2 mM EDTA, 5 mM DTT, 5% v/v glycerol, 250 mM NaCl (HKP buffer). The suspension was dialysed against excess HKP buffer, diluted 1:10 in low salt phosphate buffer, LSPB, (25 mM $KH_2PO_4/K_2HPO_4$, pH 6.5, containing 1 mM EDTA, 5 mM DTT and 5% v/v glycerol), and applied to 5 ml HiTrap SP HP and heparin HP columns assembled in tandem then washed with LSPB. The SP column was removed and the heparin column eluted with a 0–1 M NaCl gradient (200 ml) collecting 5 ml fractions. The purest fractions were identified by SDS–PAGE, dialysed overnight prior to anion exchange chromatography in 25 mM Tris–HCl, pH 8.0, containing 200 mM NaCl, 1 mM EDTA, 5 mM DTT and 5% w/v glycerol. A 5 ml HiTrap Q HP column was equilibrated in excess of Q buffer (25 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, 5 mM DTT and 5% w/v glycerol). The sample was diluted 1:10 with Q buffer prior to loading. The column was washed and eluted with a linear gradient of 0–0.25 M NaCl in 120 ml. Fractions (5 ml) were checked for the presence of 28 kDa protein by SDS–PAGE. A 5 ml HiTrap heparin HP column was equilibrated as described above, then loaded with the pooled Q fractions at 15%, against LSPB at 5 ml min$^{-1}$. The column was washed extensively then eluted with 0–350 mM NaCl, over 30 ml, followed by 350–600 mM NaCl, over 75 ml, collecting 5 ml fractions. Fractions that contained pure protein, as determined by SDS–PAGE analysis, were stored at –20°C in 50% glycerol.

### Production of glutathione-*S*-transferase-ExoIX fusion protein

*E. coli* strain BL21 (DE3) pGEX-5-x-3(*xni*) was generously provided by Dr W. A. Franklin (Albert Einstein College of Medicine, New York) and induced as previously described (25). Cell lysis (6 g cell pellet) and ammonium sulphate precipitation were performed, as described for native ExoIX, and the final ammonium sulphate pellet was re-suspended and dialysed into in 25 mM sodium phosphate, pH 7.3, containing 2 mM EDTA, 5 mM DTT and 150 mM NaCl. Clarified supernatant (40 ml) was applied to a pre-equilibrated 1 ml GSTrap column (GE healthcare), run at 1 ml min$^{-1}$. The column was washed in excess of 10 ml at 0.5 ml min$^{-1}$ and the column eluted with 5 ml 50 mM Tris–HCl, pH 8.0 containing 10 mM reduced glutathione. Samples contained the purest fractions of GST-ExoIX (50 kDa) were adjusted to 25 mM Tris–HCl, pH 8.0, filtered (0.2 μm) and applied to a 5 ml HiTrap Q anion column at 25% against Q buffer. The column was washed in excess of 50 ml with Q buffer, then eluted over a 60 ml 0–1 M NaCl gradient, at 2.5 ml min$^{-1}$, collecting 3 ml fractions. Samples containing the purest bands of the fusion protein were adjusted to 50% v/v glycerol and stored at –20°C.

### Nuclease assays

Radiolabelling of d(5′-GATGTCAAGCAGTCCTAAC TTTGAGGCAGAGTCC) was performed in a reaction (30 μl) containing 5 pmol oligonucleotide, [1x] kinase buffer (70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT), 17 pmol [γ-$^{32}$P] ATP and 30 U polynucleotide kinase at 37°C for 1 h. Reactions were quenched by heat denaturation at 80°C for 5 min. Labelled oligonucletides were purified using Sep-Pak Plus C18 cartridges, wetted with HPLC-grade acetonirile (100% ACN) and equilibrated with 10 mM Tris–HCl, pH 7.6. The labelling reaction was applied to the cartridge, washed extensively with 3% v/v ACN and eluted with 30% v/v ACN. The eluate was evaporated to dryness and the oligonucleotide re-suspended in a buffer containing 10 mM Tris–HCl, pH 7.6. Radiolabelled nuclease assays were performed with 1.5 nM labelled oligonucleotide and varying amounts of test protein (described in figure legend) in a buffer comprising of 25 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl and 0.3 mg ml$^{-1}$ acetylated BSA, incubated at 37°C for 10 min. Reaction products were separated by denaturing 15% PAGE, according to standard methods (28).

The release of acid-soluble nucleotides from high molecular weight (Type XIV; Sigma) DNA was monitored by UV spectroscopy as described, except that the assay contained DNA at 800 μg ml$^{-1}$ in 500 μl 25 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 2 mM DTT (30). A zymogram assay was used to identify nuclease activity in protein bands or spots separated by 1 (31) or 2D electrophoresis. Zymogram gels contained ca. 30 μg ml$^{-1}$ Type XIV DNA from herring testes (Sigma). After electrophoresis, the proteins were re-natured *in situ* and $MgCl_2$ added to a concentration of 10 mM. After staining with ethidium bromide, exonuclease activity was visualized as a shadow against a fluorescent background when viewed on a UV transilluminator.

### Isolation and identification of the DNase associated with ExoIX

A fraction (5 ml) adjacent to the ExoIX peak, with prominent nuclease activity and approximate molecular weight of 28 kDa, was dialysed against a 20-fold excess of wash buffer, 25 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 5% v/v. The sample was loaded onto a 1 ml Probond column (Invitrogen), washed in

excess of 20 ml with wash buffer, then protein eluted with a 0–500 mM imidazole gradient (20 ml), collecting 1 ml fractions. Fractions were assayed by *in situ* DNase activity gel and Coomassie staining methods. Protein in the column flow through (containing the ca. 28 kDa DNase activity) was precipitated by the addition of ammonium sulphate to a 4 M final concentration, sedimented by centrifugation at $35\,000 \times g$ for 30 min and the pellet re-suspended in 5 ml 20 mM Tris–HCl, pH 8.0, containing 100 mM NaCl and 5% v/v glycerol. The protein sample was concentrated 10-fold, by centrifugation at $6000 \times g$ for 30 min in a Vivaspin concentrator device (MWCO 10000) into 25 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, 5 mM DTT and 10% glycerol. The sample was then analysed by 2D zymogram and Coomassie gels.

### 2D electrophoresis

For each Immobilized pH Gradient (IPG) strip (17 cm ReadyStrip, non-linear pH 3–10, Bio-Rad Laboratories) a protein sample was made up to 300 µl with reswell buffer, RSB (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, 0.2% v/v ampholytes, pH 3–10). IPG strips were actively re-hydrated at 50 V for 16 h at 20°C in a Bio-rad Protean II IEF cell. IEF was carried out at 250 V for 15 min, linear ramping from 250 to 10 000 V over 3 h followed by 10 000 V for 60 000 V h. The second dimension was carried out on a 12.5% SDS–PAGE gel as described in Allen *et al.* (32). Analytical gels were stained with Biosafe Coomassie G250 stain or assayed for nuclease activity by *in situ* re-naturation and ethidium bromide staining. Protein spots were excised from the gel and digested with trypsin. Identification was effected using MALDI-tof generated mass spectrometry data analysed by MS-FIT and MASCOT web-based software, allowing for peptide mass tolerance of ±150 ppm, monoisotopic masses and variable oxidation modification.

### Covalent immobilization of ExoIX and pull-down assays

ExoIX or BSA (10 mg) were immobilized covalently, using 1 ml of CNBr-activated Sepharose 4B, essentially as described by the manufacturer (GE Healthcare). *Escherichia coli K12-MG1655* cell-free lysates were prepared as described above and the protein stored as ammonium sulphate slurry. A portion (1.8 g) of ammonium sulphate precipitate was re-suspended in a 5 ml final volume of buffer ABB (25 mM Tris–HCl, pH 8.0 containing 100 mM NaCl, 5% w/v glycerol, 2 mM DTT, with and without 1 mM EDTA) and dialysed twice against a 20-fold excess of ABB (7 ml after dialysis). Preparations in the absence of EDTA were treated with 200 U $ml^{-1}$ bovine pancreatic deoxyribonuclease I (DNase I) (Sigma-Aldrich). $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.5 mM) were added and the sample incubated for 1 h at room temperature. Lysate (1 ml) was added to either ExoIX or BSA-linked Sepharose 4B (~40 µg) and incubated with end-over-end rotation for 1 h at room temperature. The mixture was then centrifuged ($100 \times g$ for 5 min) and the supernatant removed (FT). The pelleted matrix was washed (4 × 1 ml ABB containing 200 mM NaCl). The final wash buffer was removed, an equal volume (ca. 40 µl) of SDS-loading buffer added and the sample prepared for electrophoresis on 12.5% SDS–PAGE. Proteins were visualized by staining with Coomassie blue. GST–ExoIX pull-downs were performed using protein derived from *E. coli* MG1655-K12. Cells (10 g) were lysed as described for ExoIX preparation and soluble proteins (50 ml) dialysed extensively against 25 mM sodium phosphate, pH 7.3, containing 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 5% w/v glycerol. A final buffer change was made into binding buffer (as above without NaCl) equilibrated for 4 h at 4°C. Soluble lysate (20 ml) was mixed with 2.0 mg of GST–ExoIX, mixed by end-over-end rotation for 1 h at 4°C before loading onto a 1 ml GSTrap column at a flow rate of 1 ml $min^{-1}$, equilibrated with 10 ml binding buffer. Flow through was re-applied to the column at the same flow rate and allowed to cycle overnight. The column was washed with 10 ml binding buffer at 0.1 ml $min^{-1}$. Bound proteins were eluted with 5 ml elution buffer (50 mM Tris–HCl, pH 8.0, containing 10 mM reduced glutathione), collecting 1 ml fractions. Controls were repeated as described above using either 1.0 mg GST or without the addition of supplementary protein.

### SSB–ExoIX crosslinking

Protein samples were prepared in 60 µl aliquots in PBS to a final concentration of 0.4 µg $µl^{-1}$ and dialysed twice against a minimum 100-fold excess of buffer. A working stock of cross-linking reagent, ethylene glycol *bis*(succinimidylsuccinate) (EGS), was freshly prepared to 50 mM concentration in DMSO, and further dilutions obtained in PBS of 5 mM and 0.5 mM. Optimization reactions (10 µl) contained 1.5 µg each protein in PBS with a final concentration of 10, 1 or 0.1 mM EGS. Reactions were allowed to proceed at room temperature for 30 min then quenched by the addition of 50 mM Tris–HCl, pH 7.5. Samples were mixed with loading buffer (5 µl) and analysed directly by SDS–PAGE and either Coomassie stained or the polyacrylamide gel was washed with 10 volumes transfer buffer (50 mM Tris, 7 mM glycine and 20% v/v methanol) for 15 min at RT and transferred to PVDF membrane using a Bio-Rad transfer cell according to the manufacturer's instructions. The PVDF membrane was blocked using 50 ml PBS-T (PBS buffer containing 0.05% v/v TWEEN 20) and 5% w/v milk powder for 1 h at RT or overnight at 4°C. The membrane was washed with 50 ml PBS-T for 30 min, followed by incubation with 0.2 µg $ml^{-1}$ primary antibody (rabbit anti-ExoIX, custom generated by York Bioscience) prepared in 20 ml PBS-T with 1% w/v milk powder at RT for 1 h. The membrane was washed with PBS-T for 30 min at RT, followed by probing with 0.2 µg $ml^{-1}$ HRP-linked secondary antibody (donkey anti-rabbit) prepared in 20 ml PBS-T with 1% w/v milk powder, incubated at RT for 1 h. A final wash was completed with PBS-T for 30 min. Blots were developed using ECL reagents supplied by GE Healthcare, according to the manufacturer's instructions.

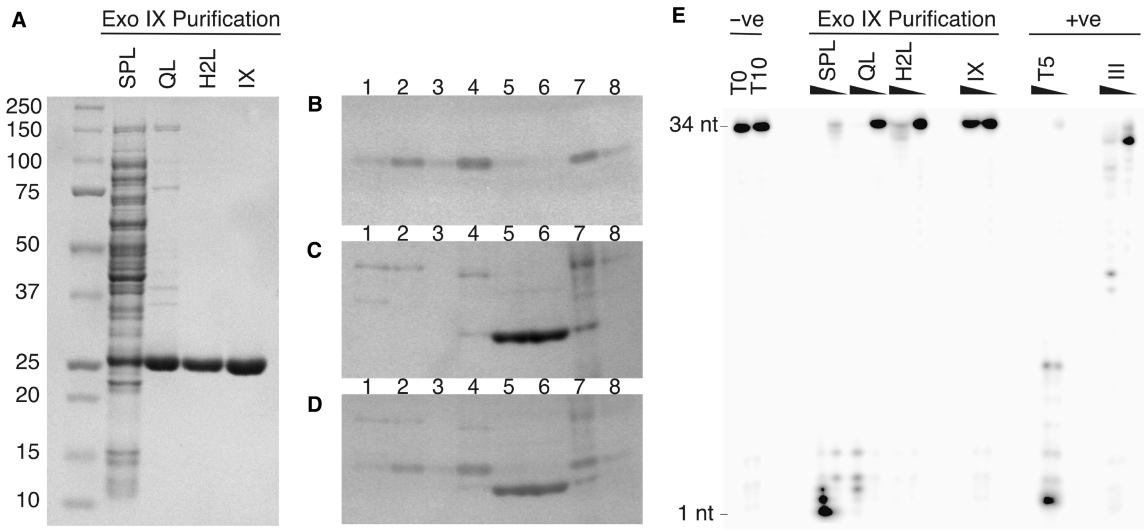


**Figure 2.** Chromatographic separation of 3′-5′ exodeoxyribonuclease activity associated with preparations of Exonuclease IX. (**A**) SDS–PAGE analysis of the purification of ExoIX from cell lysate of induced BL21 (pJONEX/*xni*, pcI857). SPL, cleared cell lysate applied to SP/first Heparin column (28 μg); QL, Q load (6 μg); H2L, second Heparin column load (5 μg); IX, concentrated ExoIX eluate from second Heparin (7.5 μg). (B–D). Eluted fractions from first Heparin column were separated by SDS–PAGE. (**B**) Ethidium bromide stained substrate gel. High molecular weight DNA cast in the gel fluoresces with UV, while regions of DNA degradation appear as darker bands. Early fractions (lanes 1–4), contain detectable exonuclease activity. (**C**) The same gel counter-stained with Coomassie G250. Over-expressed ExoIX is eluted in later fractions (lanes 5 and 6). (**D**) Superimposition of images in panels B and C, demonstrating that exonuclease activity can be resolved from ExoIX. A fraction represented in lane 4 was used for subsequent enrichment and identification of the co-purifying nuclease. Lanes, 1–6, heparin fractions (2.5 μl); 7, loading sample (5 μl); 8, flow through (5 μl). (**E**) Highly purified ExoIX lacks activity on a single-stranded DNA substrate (34-mer). Protein samples taken during the purification of ExoIX were incubated with 15 fmol $^{32}$P-labelled 34-mer at 37°C for 10 min in the presence of 10 mM $MgCl_2$ and the reaction products separated by denaturing PAGE. Reactions (10 μl) contained varying amounts of protein. SPL, 0.7 and 0.07 μg of protein from cell-free extract of induced cells expressing ExoIX; QL, 0.1 and 0.01 μg of protein loaded on to first anion exchange column; H2L, 3 and 0.3 μg of protein from sample loaded onto second heparin column; IX, contains samples from final purified fraction of ExoIX eluted from second heparin column, 5 and 0.5 μg; two positive controls are also shown, bacteriophage T5 D15 exonuclease (T5), 0.1 and 0.01 μg and exonuclease III (III), 0.03 and 0.003 μg.

### Identification of PAGE-resolved proteins

Resolved protein bands or spots were excised from polyacrylamide gels, digested with trypsin and analysed by MALDI-TOF mass spectrometry (Aberdeen Proteome Facility). Proteins were identified from peptide fragments by comparison to theoretical digests of the *E. coli* proteome *in silico*, using MASCOT (http://www.matrixscience.com/) or MS-fit search tools (http://prospector.ucsf.edu/).

## RESULTS

### Purification of DNase-free ExoIX

The *xni* gene was expressed at high levels using the heat-induced pJONEX expression system. Soluble protein was readily obtained and purified to over 98% purity as estimated by densitomety using a combination of ion-exchange resins (Figure 2A). Zymogram assays (Figure 2B) on ExoIX fractions from the initial ion exchange column revealed the presence of a DNase with a similar, but not co-incident electrophoretic mobility with the major protein band (Figure 2C). No DNase activity was observed which co-migrated with ExoIX protein. Liquid nuclease assays were carried out on samples from each stage of the natively expressed (i.e. untagged) ExoIX purification. These assays showed that the final purified fraction lacked any significant DNase activity (below 0.02 units, Table 1). Similarly, the GST–ExoIX fusion protein also appeared to lack any significant nuclease activity. Two positive controls, T5 D15 5′-3′ exonuclease (30) and exonuclease III (New England Biolabs) were also included in the assays. These proteins showed potent exonuclease activities of 614 and 227 units, respectively. A range of different divalent metal cofactors, pH and salt conditions was examined, but in no case were we able to detect any significant DNase activity in fully purified

**Table 1.** Purification of ExoIX

| Sample | Total Yield (mg) | Purity (%)[a] | Specific activity[b] |
|---|---|---|---|
| Purification of native ExoIX | | | |
| Hep/SP Load | 1032 | 12.5 | 0.18 |
| QL | 43 | 68.7 | 0.02 |
| 2nd Heparin Load | 33 | ⩾95 | ⩽0.03 |
| Heparin (F21) | 5.8 | ⩾98 | ⩽0.02 |
| GST–ExoIX fusion protein | 8 | ⩾95 | ⩽0.02 |
| Positive Controls: | | | |
| ExoIII | | | 227 |
| T5FEN (JRS) | | | 614 |

[a]Purity was estimated by densitometry of Coomassie-stained SDS-PAGE gels.
[b]Units of activity per μg of protein. One unit of enzyme is defined as that required to release 1 nmol acid soluble nucleotides in 30 min at 37°C.

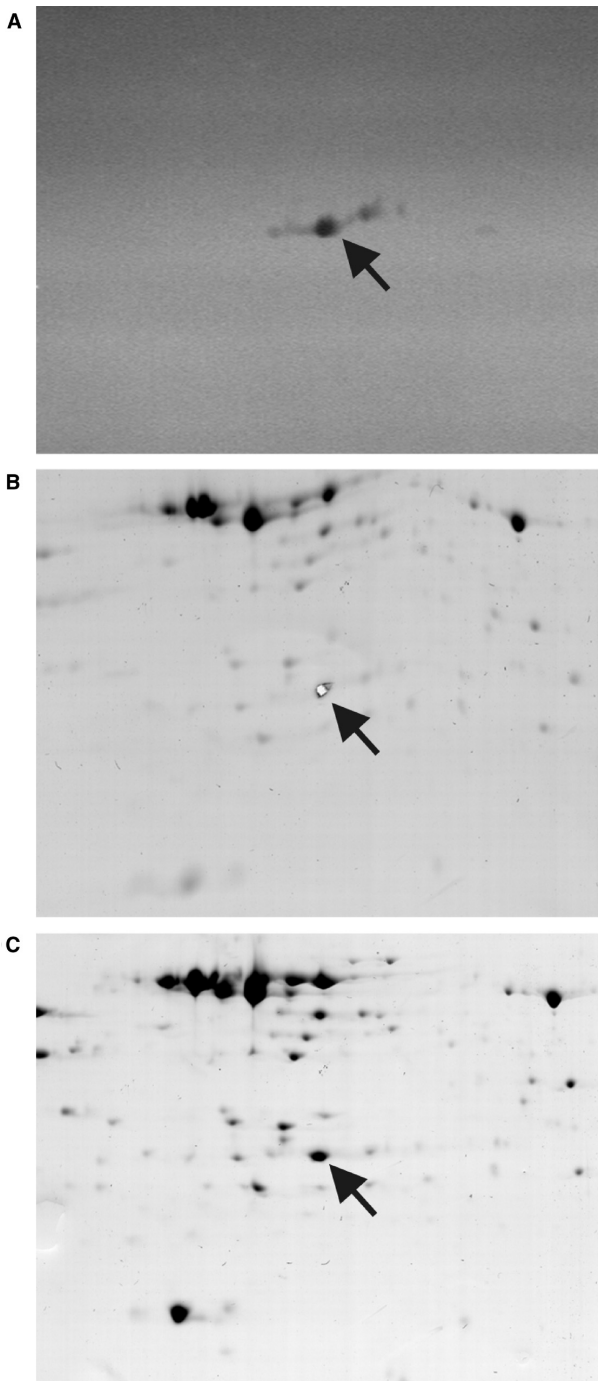


**Figure 3.** Two-dimensional SDS-polyacrylamide gel electrophoresis (12.5% gel) to isolate a contaminating DNase from partially purified cell extract of *E. coli* BL21 pJONEX/*xni* p*cI*857. Protein (80 µg) from the concentrated flow through of a Ni-chelate column was resolved by isoelectric focusing, over a pH 3-10 non-linear gradient in the first dimension and separated by molecular weight by denaturing SDS-PAGE in the second (refer to Materials and Methods for detailed experimental conditions). (**A**) Substrate gel cast with 40 µg ml$^{-1}$ type XIV herring sperm DNA, stained with ethidium bromide and visualized with UV. The dark spot is caused by DNA degradation by the co-purifying DNase. The region of activity was excised as shown (**B**), the substrate gel counter-stained with Coomassie blue. (**C**) Replicate gel stained with Coomassie alone. Arrows indicate the regions corresponding to DNase activity (pI 5.8). Excised spots were digested with trypsin and the fragments analysed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

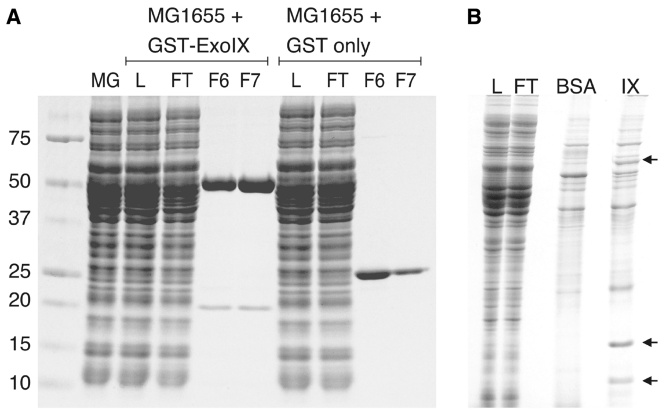


**Figure 4.** Protein interactions of ExoIX. (**A**) SDS–PAGE analysis of the protein–protein interactions of GST–Exo IX and lysate of *E. coli* MG1655. Cell free lysate of *E. coli* MG1655 was pre-incubated with either GST–ExoIX or GST for 1 h at 4°C. The mixtures were loaded onto a GSTrap column (GE Biosciences), washed and eluted with 10 mM glutathione. Of the proteins which specifically eluted with GST–Exo IX only one was visible in the final gel image (~20 kDa). Lanes, MG, MG1655 cell free lysate; L, GSTrap load; FT, flow through; F6/7, eluted fractions. (**B**) SDS–PAGE analysis of pull-down assays with ExoIX-Sepharose. MG1655 lysate was treated with DNase I, prior to incubation with immobilized protein. Arrows indicate specific protein interactants which are present in the eluate from ExoIX-Sepharose (Table 2) and absent in the BSA-Sepharose control. Abbreviations, L, MG1655 cell lysate; FT, flow through; BSA, BSA-Sepharose 4B eluate; IX, ExoIX-Sepharose eluate.

ExoIX using this assay. We then deployed a sensitive assay using radiolabelled oligonucleotides. This showed that partially purified ExoIX samples did possess a copurifying 3′-5′ exonuclease activity but it was finally separated from ExoIX by the final heparin column (Figure 2E).

### Identification of copurifying DNase

A fraction taken from a side peak from the first heparin column was analysed on a 2D SDS–PAGE zymogram gel. The result is shown in Figure 3. The spot of nuclease activity was excised from the gel and the protein identified as exonuclease III by mass spectrometry.

### Interaction of ExoIX with *E. coli* lysates

A 20 kDa protein from *E. coli* cell-free extracts was shown to interact with both covalently immobilized ExoIX and GST–ExoIX fusion proteins (Figure 4) but was not retained by either control matrix (GST or Sepharose-BSA) demonstrating that the interaction was with ExoIX and not the matrix or fusion protein affinity tag. Figure 4B also revealed bands of ~60 and 15 kDa that appeared to stick to the covalently immobilized ExoIX matrix. They were identified as histone-like nucleoid-structuring protein H-NS, the 17 kDa protein Skp, glyceraldehyde-3′-phosphate dehydrogenase and GroEL (Table 2).

### Cross-linking studies

The interaction between ExoIX and SSB was further investigated using chemical cross-linking of the over-expressed and recombinant proteins. SSB protein formed

**Table 2.** Identification of proteins interacting with ExoIX

| Nominal Mw (from gel) | Identification | Theoretical MW | Sequence coverage[a] | Significance |
|---|---|---|---|---|
| 60 000 | G-3-P dehydrogenase | 58 959 | 34% | 5.3 e + 05[b] |
| | Chaperone GroEL | 57 329 | 38% | 3.1e + 04[b] |
| 22 000 | Single-Stranded DNA Binding Protein (SSB) | 18 975 | 53% | 1.0 e + 05[b] |
| 15 000 | Seventeen kilodalton protein (Skp) | 17 677 (15 692 mature peptide) | 31% | 3.9 e-07* |
| 17 000 | Histone-like Nucleoid Structuring Protein (H-NS) | 15 456 | 47% | 0.00089** |

[a]Percentage of peptide fragments that cover the entire length of the theoretical gene product.
[b]MOWSE score (MS-Fit analysis, values >0.05 represent greater significant).
*$P$-value, the probability of finding the same score in a random database, values <0.05 are significant.
**Expect (E) value where values <0.05 are significant.

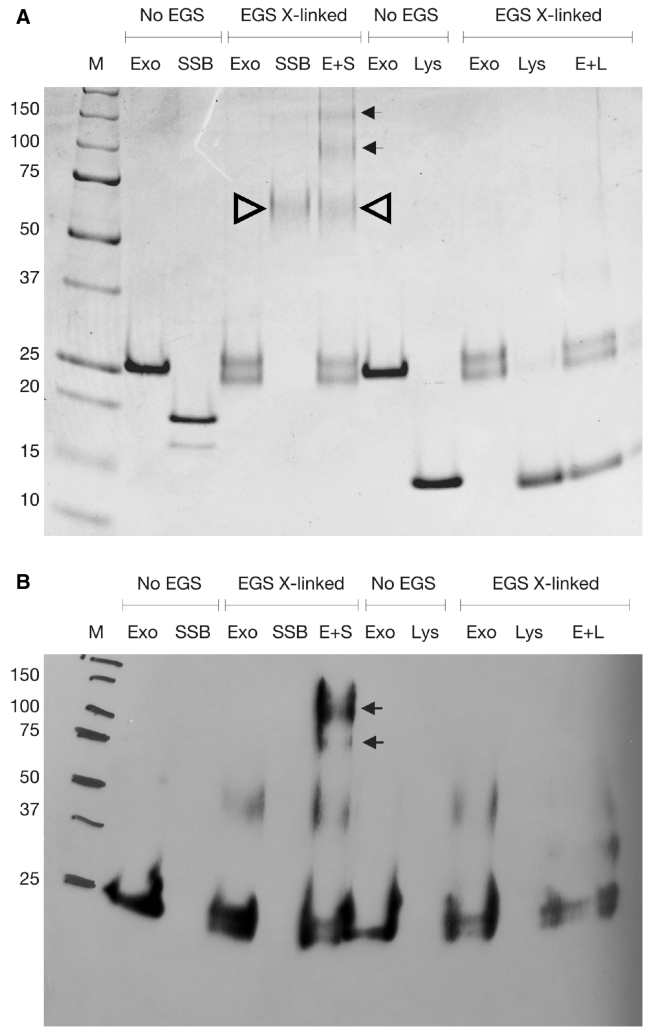


**Figure 5.** Ethylene glycol bis(succinimidylsuccinate) (EGS) cross-linking of exonuclease IX (Exo IX) and single-stranded DNA binding protein (SSB). Proteins were prepared in PBS in the presence (EGS X-linked) or absence (No EGS) of 1 mM EGS and incubated at room temperature for 30 min. Samples were separated by 10% acrylamide SDS–PAGE. (**A**) Coomassie stained gel of cross-linking. Tetrameric SSB is indicated with open triangles, ExoIX:SSB complex with black. (**B**) Western blot analysis of a replicate SDS–PAGE gel, probed with rabbit anti-ExoIX (primary antibody) and donkey anti-rabbit-HRP (secondary antibody), developed using the ECL system (GE Biosciences). The ExoIX:SSB complex is indicated with arrows. Abbreviations, Exo/E, exonuclease IX; SSB/S, single-stranded DNA binding protein; lys/L, lysozyme. E + S; ExoIX with SSB. E + L; ExoIX plus lysozyme as a negative control. Gels for Coomassie staining were loaded with 2.6 μg protein/lane and for westerns with 0.7 μg protein/lane.

the expected multimer migrating close to 70 kDa when incubated alone, but when ExoIX was present two migrating species of ~100 and 150 kDa were clearly seen (Figure 5A) on SDS–PAGE analysis. Western blotting followed by detection with anti-ExoIX polyclonal antibodies confirmed that the two higher species contained ExoIX (Figure 5B). The cross linking was performed on DNA-free samples indicating that this is a direct interaction between proteins.

## DISCUSSION

The first reported biochemical study on ExoIX was performed using a glutathione-*S*-transferase fusion protein. It suggested that ExoIX possessed 3′-5′ exonuclease activity and a 3′-phosphodiesterase activity on DNA with a 3′-incised abasic lesion. Neither activity was quantified in terms of turnover number nor was the enzyme purified to constant specific activity (25). As can be seen in Figure 2, stubborn nuclease activity was present during the early stages of purification, but was finally eliminated after extensive ion exchange chromatography. Here we have conclusively shown that the DNase activity associated with ExoIX is due to partially copurifying exonuclease III. The nature of the contamination was confirmed by direct identification of the spurious nuclease from a 2D substrate gel.

We have previously observed contaminating nuclease activity initially copurifying with overexpressed proteins. Though weak, such contaminating activities are readily detected using sensitive radioisotopic assays such as those deployed in the previous ExoIX study. We repeated the purification of a GST–ExoIX fusion protein using a column format rather than the originally employed batch recovery method (25). Extensive washing and affinity elution resulted in a protein that lacked any detectable DNase activity as was the case with the unmodified ExoIX protein. These results are intriguing given that the amino acid sequence of ExoIX possesses the ligands equivalent to those responsible for binding the catalytically essential metal ion in the FENs (19).

We investigated whether ExoIX interacted directly with any other *E. coli* proteins such as exonucelase III. Using a pull-down format or column chromatography approaches we were unable to reproducibly show any strong interaction with exonuclease III however, several *E. coli* proteins did appear to be able to bind to ExoIX. Both the native

and the GST–ExoIX fusion proteins were able to bind to *E. coli* SSB. SSB forms a tetramer and is known to bind tightly to single-stranded DNA within the *E. coli* cell. ExoIX appears to be able to bind to the SSB tetramer as two extra species are observed in the cross-linking mixture of SSB and ExoIX compared with SSB or ExoIX alone. Both the slowest migrating species in the ExoIX-SSB reaction reacted with the anti-ExoIX polyclonal antibodies. It should be noted that it is difficult to accurately determine the stoichiometries of binding of cross-linked proteins by SDS–PAGE analysis. However, as two slower migrating SSB–ExoIX complexes can be observed, the simplest explanation is that each SSB tetramer can bind two ExoIX molecules. We were also able to detect binding of ExoIX to the H-NS protein, a molecule with a preference for binding to curved duplex DNA and G/C-rich single-stranded nucleic acids (33,34). These results indicate that ExoIX is involved in someway with the replication machinery, though despite our extensive efforts we have been unable to identify any substrate on which ExoIX displays hydrolytic activity. We also identified chaperones GroEL, GroES and Skp but these may have been binding to ExoIX that had become partially denatured during the immobilization process, as a common role for chaperones is to bind disordered peptides. The presence of glyceraldehyde-3-phosphorylase was almost certainly due to co-migration on SDS–PAGE of this protein with the chaperone GroEL as they share very similar molecular masses.

This study reveals that ExoIX is not a 3′-5′ exonuclease as previously suggested (25), yet it does seem to interact intimately with the DNA binding proteins SSB and H-NS. The homology of the protein with the FEN family of structure-specific nucleases suggests a role in processing of nucleic acids. Studies on an *xni* null mutant failed to identify any obvious defects in a range of DNA repair pathways (35). However, as ExoIX is a paralogue of the FEN domain of DNA Pol I, perhaps it functions as a back-up for this enzyme, as previously suggested (21). Further studies are needed to determine the biological role of this cryptic DNA Pol I paralogue.

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