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DNA deoxyribophosphodiesterase of *Escherichia coli* is associated with exonuclease I

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ABSTRACT

DNA deoxyribophosphodiesterase (dRpase) of *E.coli* catalyzes the release of deoxyribose-phosphate moieties following the cleavage of DNA at an apurinic/apyrimidinic (AP) site by either an AP endonuclease or AP lyase. Exonuclease I is a single-strand specific DNA nuclease which affects the expression of recombination and repair pathways in *E.coli*. We show here that a major dRpase activity in *E.coli* is associated with the exonuclease I protein. Highly purified exonuclease I isolated from an over-producing stain contains high levels of dRpase activity; it catalyzes the release of deoxyribose-5-phosphate from an AP site incised with endonuclease IV of *E.coli* and the release of 4-hydroxy-2-pentenal-5-phosphate from an AP site incised by the AP lyase activity of endonuclease III of *E.coli*. A strain containing a deletion of the *sbcB* gene showed little dRpase activity; the activity could be restored by transformation of the strain with a plasmid containing the *sbcB* gene. The dRpase activity isolated from an overproducing stain was increased 70-fold as compared to a normal *sbcB*$^+$ strain (AB3027). These results suggest that the dRpase activity may be important in pathways for both DNA repair and recombination.

INTRODUCTION

One of the most common forms of damage to DNA is the loss of a base, forming either an apurinic or apyrimidinic (AP) site. AP sites occur in DNA as a result of the enzymatic repair of damaged bases by glycosylases (1), and can arise spontaneously in DNA by depurination (2). They can also occur as a result of exposure to ionizing radiation (3). The AP site itself is both a cytotoxic and mutagenic lesion (4,5) and cells contain repair enzymes for their removal. In *E.coli*, at least four enzymes are thought to be responsible for the repair of AP sites. First, an AP endonuclease such as exonuclease III or endonuclease IV introduces a single-strand break on the 5′ side of the base-free deoxyribose-phosphate moiety through hydrolysis of the phosphodiester bond. Next, DNA deoxyribophosphodiesterase (dRpase) removes the deoxyribose-phosphate moiety remaining at the site creating a nucleotide-free site (6). DNA polymerase I is then able to replace the absent nucleotide, which can then be sealed at its 3′ end to the rest of the DNA molecule by the action of DNA ligase. Such a pathway predicts a very short patch repair of one nucleotide, which has been demonstrated recently for the repair of uracil residues in DNA (7).

The removal of the deoxyribose-phosphate moiety by a dRpase activity is necessary, as it has been shown previously that this group blocks the 5′→3′ exonuclease activity of DNA polymerase I (6,8). In *E.coli*, this activity has been found in a protein with a molecular weight from 50–55 kDa that requires Mg^{++} for activation (6). The enzyme excises 2-deoxyribose-5-phosphate moieties at incised AP sites and does not have an endogenous AP endonuclease activity or exonuclease activity acting on double-stranded DNA (6). An analogous activity has been recently isolated from mammalian cells (9).

We demonstrate here that a major DNA dRpase activity of *E.coli* is associated with the enzyme exonuclease I. Exonuclease I was originally shown to be an exonuclease that degrades single-stranded DNA in the 3′→5′ direction (10,11). It was later identified as the structural gene for the *sbcB* locus by analysis of supressors of *recB* and *recC* (exonuclease V) mutations (12). The *recB* and *recC* mutants are recombinationally deficient and show sensitivity to DNA damaging agents such as UV light and mitomycin C (13). The absence of exonuclease I activity in *recB* and *recC* mutants restores both genetic recombination and resistance to UV light and mitomycin C to near wild-type levels (12). Another class of alleles, *xonA*, show only a suppression of the UV light and mitomycin C sensitivities but remain recombinationally deficient (14). The role of the dRpase activity as a function of exonuclease I and its effects on recombination and DNA repair will be considered.

MATERIALS AND METHODS

Bacterial Strains and Lysates

The following *E.coli* K-12 strains were used: AB3027 (*xth polA*) (15), KL148 (Δ(*sbcB-his*17) supplied by Dr. Barbara Bachmann from the Yale University *E. Coli* Genetic Stock Center, and SK7743, containing a plasmid that overexpresses the sbcB gene product (JC8679 + pDPK20) (16), a generous gift from Dr.

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Sidney Kushner, University of Georgia. Bacteria were grown as 300 ml cultures in Luria broth to late log phase (with 20 μg/ml ampicillin for SK77743), collected by centrifugation, and were washed once in extraction buffer (50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM DTT, 1 mM NaEDTA, 5% glycerol). After centrifugation, the cells were suspended in 3 ml of the extraction buffer. The cells were disrupted by sonication and debris was removed by centrifugation at 10,000 g for 30 min (fraction I). Protein concentrations were determined by the Coomassie-blue method (17). Strain KL148 was transformed with the plasmid pDPK20, isolated from the strain SK7743, as described previously (18).

Enzymes and Reagents

E.coli uracil-DNA glycosylase (19) and endonuclease IV (20) were prepared as described previously. Endonuclease III was a generous gift of Dr. Richard Cunningham, State University of New York, Albany. Exonuclease I and the large-fragment (Klenow) of DNA polymerase I were purchased from US Biochemicals. Poly(dA-dT) and DNA polymerase I were purchased from Boehringer Mannheim. DNase I was purchased from Sigma. DEAE Sepharose fast-flow and Heparin Sepharose CL-6B were purchased from Pharmacia.

Polynucleotide Substrates and Enzyme Assays

Poly(dA-dT) containing ^{32}P-labeled AP sites was prepared as described previously (6,9). The poly(dA-dT) containing 5′ [^{32}P] dUMP residues following the incorporation of [α-^{32}P] dUTP by the action of the Klenow fragment was treated with uracil-DNA glycosylase and then with either endonuclease IV to generate a substrate incised at the 5′ side of an AP site or with endonuclease III to generate a substrate cleaved on the 3′ side of an AP site. DNA dRpase activity is assayed in a standard reaction measuring the release of 2-deoxyribose-5-phosphate (for 5′ incised AP sites) (6) or 4-hydroxy-2-pentenal-5-phosphate (for 3′ incised AP sites (9, 21,22). Reaction mixtures (100 μl) contained 50 mM Hepes-KOH, pH 6.5, 10 mM $MgCl_2$, 5 mM DTT, poly(dA-dT) containing incised AP sites (3000 cpm) and a limiting amount of enzyme. After incubation at 37°C for 30 min, release of sugar phosphate is determined either by precipitation with trichloroacetic acid in the presence of Norit charcoal (6) or by direct injection of the reaction mixture onto a Brownlee MPLC AX (4.6 mm×3 cm) HPLC column eluted with 25 mM KH_2PO_4, pH 3.5, at a flow rate of 1 ml/min. One unit of dRpase activity is defined as the release of 1 pmol of 2-deoxyribose-5-phosphate at 37°C in 30 min.

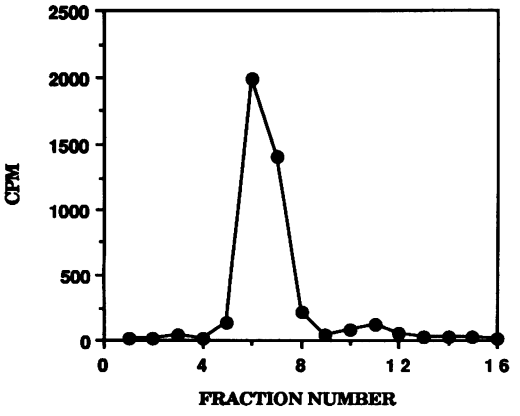

Figure 1. Enzymatic release of 2-deoxyribose-5-phosphate from a [^{32}P]-labeled poly(dA-dT) substrate containing 5′ incised AP sites following treatment with exonuclease I. The reaction products were resolved on a MPLC AX HPLC column. 2-deoxyribose-5-phosphate elutes between fractions 6 & 7 (3.5 min) under these conditions (6).

The lambda DNA substrate was prepared by a nick-translation reaction. Lambda DNA (1 μg) was incubated in a reaction mixture (50 μl) of 10 units of DNA polymerase I, 0.01 ng of DNase I, 10 μM dATP, 10 μM dCTP, 10 μM dGTP, 1 μM dTTP, 10 μM dUTP, 2 μCi [α-^{32}P] dUTP, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM DTT for 1 hr at 16°C. The reaction was stopped by the addition of 2 μl of 0.5 M NaEDTA and by heating at 65°C for 5 min. The labeled DNA was separated from unincorporated nucleoside triphosphates on a Nick G-50 column (Pharmacia) and was subsequently precipitated with ethanol and lyophilized. It was then treated with uracil-DNA glycosylase and endonuclease IV as for the poly (dA-dT) substrate (6,9).

Enzyme Purification

The crude bacterial cell lysate was passed through a DEAE Sepharose fast-flow column (1×2.5 cm) that had been equilibrated with extraction buffer (fraction II). To 4 ml of faction II, 1.3 g of $(NH_4)_2SO_4$ was added to 50% saturation and after 15 min of stirring at 4°C, the mixture was centrifuged at 10,000 g for 20 min. The pellet was suspended in 1 ml of 20 mM Tris-HCl, pH 7.8, 5 mM DTT, 1 mM NaEDTA (buffer A, fraction III). An aliquot of fraction III (2.6 mg) was first loaded onto a NAP-5 column to remove residual $(NH_4)_2SO_4$ and was eluted with 1 ml of buffer A. This material was then loaded onto a Heparin Sepharose CL-6B column (1×2.1 cm) and was washed with 12 ml of buffer A and eluted with a 20 ml gradient from 100% buffer A to 100% buffer A containing 1 M NaCl at a flow rate of 1 ml/min.. Fractions were collected every min and were assayed for dRpase activity. For gel filtration, an aliquot of fraction III (3 mg) was loaded onto a Superdex 75 HR 10/30 FPLC column (Pharmacia) previously equilibrated with 50 mM Tris-HCl, pH 7.8, 5 mM DTT, 1 mM NaEDTA, 100 mM NaCl, 5% glycerol (buffer B). The column was eluted with buffer B at a flow rate of 0.5 ml/min, and fractions were collected every 0.5 ml.

RESULTS

DNA dRpase Activity in a Highly Purified Preparation of Exonuclease I

Exonuclease I of *E.coli* was originally demonstrated to be a single-strand specific enzyme that digests DNA in the 3′→5′ direction (10,11). It is most active at pH 10.5. The protein contains 465 amino acids with a molecular weight of 53,174 Da, and the structural gene (*sbcB*) has been cloned and sequenced (23,24). A highly purified preparation of exonuclease I (>95% purity), isolated from an *E.coli* strain that overproduces the enzyme, was obtained from a commercial source and tested for dRpase activity.

Figure 1 shows the release of 2-deoxyribose-5-phosphate from a polynucleotide substrate containing AP sites incised at the 5′ side by endonuclease IV. Radioactivity released from the polynucleotide substrate co-elutes with 2-deoxyribose-5-phosphate when the reaction products are resolved on anion exchange HPLC. In figure 2, the pH dependence of enzymatic release of

2-deoxyribose-5-phosphate is shown. As with previous preparations of DNA dRpase, maximal release of sugar-phosphate was seen between pH 6.5 and 7.0 (6,25).

In figure 3, the release of the product 4-hydroxy-2-pentenal-5-phosphate from a polynucleotide substrate containing AP sites incised at the 3′ side by the AP lyase endonuclease III was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal (6,9,25). Endonuclease III is one of several glycosylases that have an associated AP lyase activity that cleaves DNA at AP sites via a β-elimination reaction, resulting in the formation of an α,β-unsaturated sugar-phosphate product at the 3′ termini (21,26). Exonuclease I actively releases the 4-hydroxy-2-pentenal-5-phosphate product.

To confirm that the apparent dRpase activity associated with exonuclease I was not specific for a poly (dA-dT) polynucleotide substrate, an analogous substrate was prepared by nick-translation of phage lambda DNA with [α-^{32}P] dUTP and subsequently treated with uracil-DNA glycosylase and endonuclease IV. This substrate was then treated with exonuclease I and the release of 2-deoxyribose-5-phosphate was measured. The apparent K_m for the release of 2-deoxyribose-5-phosphate was determined by Lineweaver–Burk analysis as shown in figure 4, and found to have the value of 0.22 μM. This value is similar to a previously measured value of K_m (0.17 μM), using a poly(dA-dT) substrate containing 5′ incised AP sites and a partially purified dRpase preparation (25).

Lack of DNA dRpase Activity in a *sbcB* Deletion Mutant

In order to confirm that the DNA dRpase activity was associated with the exonuclease I gene product, a deletion mutant in *sbcB* was obtained and the dRpase activity was isolated following separation on Heparin Sepharose or gel filtration. Figure 5 shows the fractionation of cell lysates prepared from strain AB3027, which is known to contain dRpase activity (6), SK7743, an overproducer of exonuclease I, and strain KL148, which is a deletion mutant of *sbcB*, on a Heparin Sepharose column. As

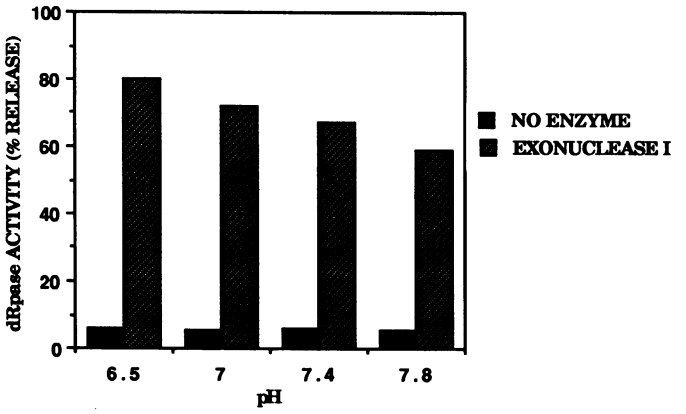

Figure 2. The pH Dependence of the enzymatic release of 2-deoxyribose-5-phosphate from a [^{32}P]-labeled poly(dA-dT) substrate containing 5′ incised AP sites by exonuclease I. DNA dRpase activity was assayed under standard conditions with 50 mM Hepes-KOH at pH 6.5, 7.0, 7.4, and 7.8. Release of 2-deoxyribose-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal.

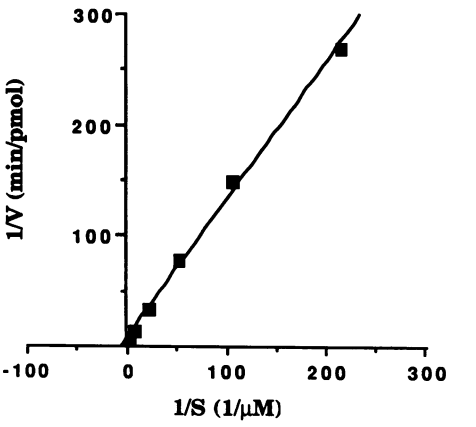

Figure 4. Lineweaver–Burk plot for the determination of K_m for the release of 2-deoxyribose-5-phosphate from a lambda DNA substrate containing 5′ incised AP sites. Substrate range, 0.005 μM to 0.24 μM; K_m = 0.22 μM.

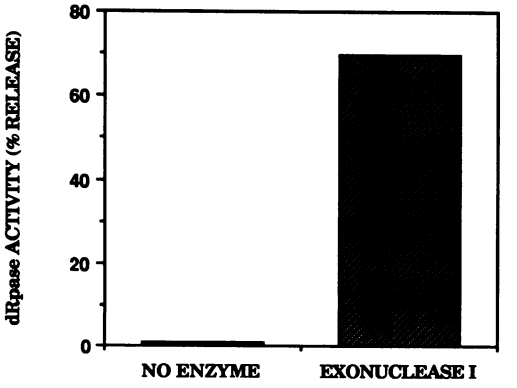

Figure 3. Enzymatic release of 4-hydroxy-2-pentenal-5-phosphate from a [^{32}P]-labeled poly(dA-dT) substrate containing 3′ incised AP sites following treatment with exonuclease I. The poly(dA-dT) substrate was treated with the AP lyase endonuclease III of *E.coli*. Release of 4-hydroxy-2-pentenal-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal.

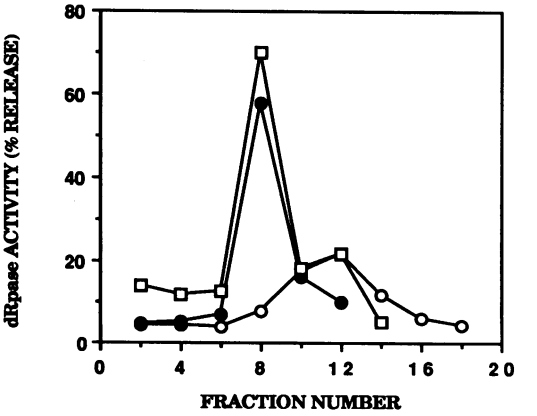

Figure 5. Heparin Sepharose chromatography of bacterial cell lysates. Release of 2-deoxyribose-5-phosphate from a [^{32}P]-labeled poly(dA-dT) substrate containing 5′ incised AP sites was assayed by precipitation with trichloroacetic acid in the presence of Norit charcoal. Strains: AB3027 (●), KL148 (○), SK7743 (□).

seen in figure 5, a large peak of dRpase activity is detectable in AB3027 and SK7743 (fraction 8) that is absent in KL148. Single-strand DNA exonuclease activity is also present in the fractions with corresponding dRpase activity (data not shown).

In figure 5 a decreased amount of sugar-phosphate releasing activity is seen in fractions that elute later than those containing the majority of the dRpase activity (fraction 12). This residual activity may be due to the presence of the fpg protein, the product of the *mutM* gene (27,28). It has been recently shown that the fpg protein, a 30.2 kDa DNA glycosylase that recognizes modified bases such as 8-oxoguanine and formamidopyrimidine also has an intrinsic dRpase-like activity (29); however, unlike DNA dRpase, this activity appears to be active in the presence of EDTA and appears to catalyze the release of sugar-phosphate moieties via a β-elimination reaction. The activity may also be a function of another unknown enzyme having an intrinsic dRpase activity.

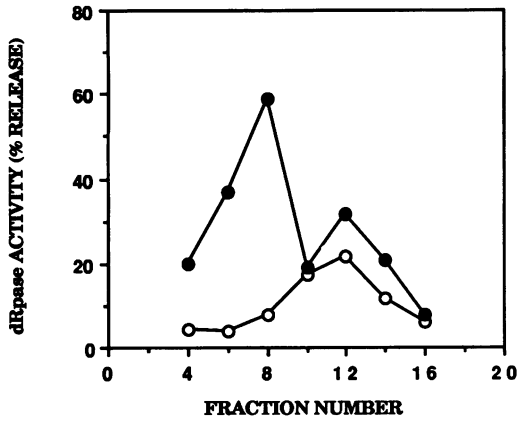

Figure 6. Heparin Sepharose chromatography of bacterial cell lysates from strain KL148 (Δ*sbcB*) (○) and strain KL148 transformed with the plasmid pDPK20 (●). Release of 2-deoxyribose-5-phosphate from a [^{32}P]-labeled poly(dA-dT) substrate containing 5′ incised AP sites was assayed by precipitation with trichloroacetic acid in the presence of Norit charcoal.

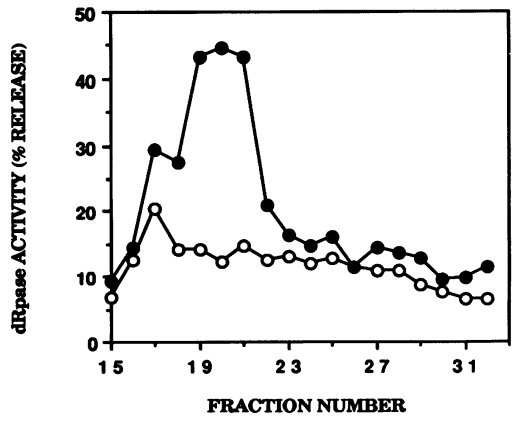

Figure 7. Superdex 75 gel filtration of bacterial cell lysates from strain KL148 (Δ*sbcB*) (○) and strain KL148 transformed with the plasmid pDPK20 (●). Release of 2-deoxyribose-5-phosphate from a [^{32}P]-labeled poly(dA-dT) substrate containing 5′ incised AP sites was assayed by precipitation with trichloroacetic acid in the presence of Norit charcoal.

To confirm the association of the dRpase activity with the *sbcB* gene product, the strain KL148 was transformed with plasmid pDPK20 that overproduces exonuclease I. As seen in figure 6, the major dRpase activity is restored, as determined by separation of cell lysates on Heparin Sepharose. The restoration of dRpase activity is also evident when the lysates were fractionated on a Superdex 75 FPLC gel filtration column (figure 7). The molecular weight of the major dRpase activity seen is between 45−55 kDa, which is in the expected range for exonuclease I.

DNA dRpase Activity in a Exonuclease I Overproducer

To further confirm the association of the dRpase activity of the exonuclease I protein, cell lysates were prepared from a strain containing a plasmid that overproduces the *sbcB* gene product. The lysates were fractionated on Heparin Sepharose, and the total units of dRpase activity was determined for the overproducer strain SK7743 and the strain AB3027. As seen in table I, there is a 70-fold increase in the enzymatic specific activity of dRpase in the overproducer. These results strongly suggest that dRpase is an activity of the *sbcB* gene product.

DISCUSSION

We have demonstrated that the sugar-phosphate releasing activity acting on both 5′ and 3′ incised AP sites is associated with the enzyme exonuclease I. Hence it appears that exonuclease I is a multifunctional enzyme that acts both as a single-strand specific exonuclease as well as an enzyme that removes single sugar-phosphate groups at termini remaining at incised AP sites. The enzyme is unique in its ability to excise sugar-phosphate groups from both 5′ and 3′ incised AP sites; it can remove both the deoxyribose-phosphate moiety as well as the α,β-unsaturated compound 4-hydroxy-2-pentenal-5-phosphate. We also have further evidence that exonuclease I is capable of removing both phosphoglycoaldehyde and phosphoglycolate moieties at 3′ termini in DNA (manuscript in preparation).

It was reported previously that a mutant in exonuclease I contained dRpase activity (6). Several lines of reasoning can explain these results. First, these original dRpase assays were performed with crude lysates and not with partially purified protein. Crude lysates can contain compounds such as polyamines that can induce the release of deoxyribose-phosphate by a β-elimination reaction (9,30−32). Second, the recent finding that the fpg protein contains a second dRpase-like activity also complicates the assays in crude lysates (29). Third, it is possible that other dRpase-like activities are present in *E.coli*.

The role of the DNA dRpase activity in the base-excision repair pathway appears to repair AP sites by allowing a DNA polymerase to properly replace a missing nucleotide which can be subsequently ligated to the DNA molecule. The presence of a dRpase-like activity associated with the fpg protein suggests that *E.coli* may have more than one function capable of removing sugar-phosphate groups at incised AP sites. This result is not

Table I. DNA dRpase activity following Heparin Sepharose Chromatography.

Strain	Protein (mg)	Specific Activity (Units/mg)	Total Activity (Units)
AB3027	0.074	1360	101
SK7743	0.080	94270	7542

totally unexpected given the redundancy of many DNA repair enzymes found in bacteria. For example, both exonuclease III and endonuclease IV are both 5′ AP endonucleases that can also remove 3′ phosphoglycolate and 3′ phosphoglycoaldehyde groups from DNA (33,34).

A *sbcB* mutant in a recBC$^+$ background does not appear to be sensitive to UV radiation or mitomycin C (35). A mutation in *mutM* that codes for the fpg protein is a mutator that leads specifically to G·C→T·A transversions (27,36). It will be of interest to expose a *sbcBmutM* double mutant to agents that damage DNA to determine if inactivation of both gene products will cause increased sensitivity to the damaging agents.

As stated previously, two types of mutants in exonuclease I have been characterized in a *recBC* background: *sbcB* and x*onA* (12,14). These mutants have been mapped and levels of exonuclease I activity (single-strand specific exonuclease) were determined for both types of mutants (37). However, the phenotypes of these mutants could not be completely explained on the basis of exonuclease I activity alone; it has been suggested that exonuclease I must contain additional functions (37,38). It will be necessary to assay several *xonA* and *sbcB* mutant strains in a *recBC* background to determine if perhaps the DNA dRpase activity is responsible for the phenotypes described.

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