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Mutations Simultaneously Affecting Endonuclease II and Exonuclease III in *Escherichia coli**

(*xth* mutations/alkylation/DNA repair)

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ABSTRACT **We studied mutants of *E. coli* originally identified as being deficient in either endonuclease II (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.30) or exonuclease III [deoxyribonucleate (double-stranded) 5′-nucleotidohydrolase, EC 3.1.4.27] activity. Twelve independently derived mutants were tested, including three new endonuclease II mutants. Deficiency of one enzyme was always accompanied by deficiency of the other. Furthermore, temperature-sensitivity of one activity was always accompanied by temperature-sensitivity of the other, and the enzymes were co-purified. The results suggested a physical association between exonuclease III and endonuclease II, which may be of advantage in the excision-repair of DNA.**

A thermolabile endonuclease II was purified from one of the new mutants, indicating that it had an altered structural gene. This mutation, and all similar ones mapped by genetic transduction, was located between the *pncA* and *aroD* genes on the *E. coli* chromosome.

One mutant had a prolonged generation time, an increased sensitivity to the alkylating agents methylmethanesulfonate and mitomycin C, and a decreased plating efficiency for bacteriophage λ, but no marked sensitivity to ultraviolet or γ-irradiation. Its enzymatic and biological abnormalities were simultaneously revertible, suggesting they were caused by a single mutation. These results suggested a role for these enzymes in normal cell growth processes and in the repair of alkylation damage.

Endonuclease II (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.30) of *Escherichia coli* introduces single strand breaks into DNA that has been partially depurinated or modified by alkylating agents like (methylmethanesulfonate ($MeSO_2OMe$) (1–4). This property suggests that it might function in an excision-repair system for such lesions in DNA. To verify its role *in vivo*, we have isolated three mutants with reduced levels of this enzyme (5). They were identified within a highly mutagenized population through enzyme assays performed on 8000 randomly chosen clones with special micro-assay screening techniques (6). We sent one of these mutants to Dr. Marcus Rhoades, who planned to use it as a source of purified exonuclease III [deoxyribonucleate (double-stranded) 5′-nucleotidohydrolase, EC 3.1.4.27]. He soon informed us, however, that it was deficient in exonuclease III as well. We then surveyed our other endonuclease II mutants as well as the exonuclease III mutants we had previously isolated (7), and we found that every strain was defective in both enzymatic activities. In this paper, we describe these experiments together with a preliminary biological and genetic characterization of the three new endonuclease-II-deficient mutants.

Abbreviations: $MeSO_2OMe$, methylmethanesulfonate; *xth*, gene symbol for exonuclease III (7, 13, 14).

* This is paper III in the series "Mutants of *Escherichia coli* with altered deoxyribonucleases." Paper II is ref. 10. Part of this work appeared in an abstract (5).

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MATERIALS AND METHODS

DNA Preparations. Radiolabeled T4 phage DNA was prepared by the methods of Thomas and Abelson (8). DNA gels (DNA entrapped in polyacrylamide) were prepared by the method of Melgar and Goldthwait (9). Alkylation of DNA gels and DNA solutions was performed with $MeSO_2OMe$ as described by Paquette *et al.* (2).

Screening Assay for Endonuclease II Mutants. The assay, based on that of Friedberg and Goldthwait (1), measured the release of DNA fragments from $MeSO_2OMe$-treated DNA gels. Plastic depression plates containing 96 wells were used for the growth and assay of 8000 mutagenized clones as previously described (6). Each clone was grown to saturation at 25° in a droplet (25 μl) of broth, centrifuged, and lysed with 50 μl of a lysozyme–EDTA mixture (6). One drop (90 μl) of a DNA gel suspension was added to each well via a 4 mm inside diameter glass tubing. Each drop contained 2–3 nmol of alkylated T4[^{32}P]DNA (1 to 4 × 10^4 cpm/nmol) in a gel suspended in 50 mM Tris·HCl buffer (pH 8.0), 6.6 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. After 30 min at 42°, 0.15 ml of 0.1 M Na_3EDTA was added, and the plates were centrifuged. Samples (15 μl) of each supernatant were removed via a manifold of 12 glass capillary tubes (6) fitted with a manifold of wire plungers (Drummond Scientific Co.). The radioactivity in each sample was determined semiquantitatively by autoradiography (6). Wild-type cells released about 20% of the radioactivity, and the mutants released 2–10%.

Quantitative Enzyme Assays. Extracts were prepared for enzyme assays from exponentially growing cells which were grown, harvested, washed, and disrupted by sonication as previously described (7). Endonuclease II was measured with the DNA gel assay of Friedberg and Goldthwait (4) using a more heavily alkylated substrate (2). Reaction mixtures contained 1 mM 2-mercaptoethanol and 50 mg/ml of bovine serum albumin as additional components. One unit of enzyme catalyzes the release of 1 nmol of DNA from an alkylated DNA gel in 30 min at the specified temperature. In one ex-

TABLE 1. *Endonuclease II activities of mutant extracts*

Strain	Grown at 25°		Grown at 42°	
	Assayed at 25°	Assayed at 37°	Assayed at 25°	Assayed at 37°
	(units/mg of protein)			
AB1157	31	63	33	48
BW2001	9	5	6	6
BW2002	30	37	19	19
BW2003	41	41	24	17

periment (Table 2) endonuclease II activity was assayed by the release of acid-soluble fragments from a solution of $MeSO_2OMe$-treated DNA. Except for the physical state of the substrate, reaction conditions were the same as for the DNA gel assay; acid-soluble radioactive material was determined as for the assay of exonuclease III (7). Exonuclease III was measured by its DNase activity on sonicated phage T7 DNA (7).

Bacterial Strains. The parent strain for the mutants was AB1157 (7), a multiple auxotroph of *E. coli* K-12. It was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and propagated at 25° to allow the survival of cells containing mutations that are lethal at high temperature (7). Strains AB3027 and AB2463 were obtained from the *Coli Genetic Stock Center*, Yale University School of Medicine, as was the transductional recipient AB1360 (*aroD6*). Strain W3899-*nam*11 (*pncA*$^-$) was obtained from Dr. A. B. Pardee and used as a transductional recipient.

Sensitivity to Alkylating Agents and Irradiation. Cells grown in nutrient broth (7) at 42° were tested at 42° while in the exponential phase of growth. Sensitivities to γ-irradiation in oxygenated cultures, to ultraviolet irradiation, and to mitomycin C were determined as described previously (7). For determination of $MeSO_2OMe$-sensitivity, cells growing in a nutrient broth were centrifuged and resuspended in minimal medium (7). They were incubated for 20 min in the absence of $MeSO_2OMe$ and then up to 90 min in its presence before being diluted and plated on nutrient agar (7). In the absence of $MeSO_2OMe$, survival was >90% for all strains tested.

Other Methods. Bacterial growth was measured turbidometrically. The following procedures were previously described: protein determinations (7), DNA determinations (7), genetic transduction (10), bacteriophage titration (7), and assays for recombinational proficiency (10).

RESULTS

Mutant Isolation. Three endonuclease II mutants of independent origin were found among 8000 clones of mutagenized cells (Table 1). At 37°, strain BW2001 had 8% of normal enzyme activity (Table 1). Strains BW2002 and BW2003 appeared to have temperature-sensitive endonuclease II activity; when grown and assayed at 25°, they were as active as their parent (AB1157), but when assayed at 37° they were only 59–65% as active. Growth at 42° reduced their enzyme levels even more, indicating that the mutant enzymes are temperature-sensitive *in vivo* as well as *in vitro* and that their thermal inactivation is not readily reversible. In a separate experiment, the activities of AB1157 and BW2001 extracts were >90% additive in mixtures; therefore, the defectiveness of BW2001 is not due to an inhibitor present in excess. We then proceeded to identify the affected DNase as endonuclease II by purification of an altered (temperature-sensitive) enzyme.

TABLE 2. *Purification of endonuclease II*

Fraction	Specific activity at 25° (units/mg of protein)		$Q_{25°}^{37°}$*	
	Wild type	Mutant	Wild type	Mutant
I. Crude	6	7	1.9	1.0
II. Streptomycin	9	14	1.9	0.8
III. Ammonium sulfate	14	10	2.2	1.0
IV. Phosphocellulose	87	78	1.4	0.5
V. Sephadex G-100	315	30	1.4	0.45
VI. DNA-cellulose	3950	835	0.65	0.35

Endonuclease II was purified by the procedure of Hadi *et al.* (11) from strains AB1157 (wild type) and BW2002 (mutant). Enzyme activities were measured by the acid-solubility assay.
* $Q_{25°}^{37°}$ = ratio of activity at 37° to that at 25°.

Purification of a Temperature-Sensitive Endonuclease II. Endonuclease II was isolated from the parent strain AB1157 and from the mutant BW2002 (Table 2). The wild-type enzyme was purified 660-fold with a yield of 10%, whereas the less stable mutant enzyme was purified 130-fold with a yield of 2%. While the wild-type enzyme decreased in stability during the later purification steps, corresponding fractions of the mutant enzyme were always more temperature-sensitive (Table 2). Samples of fraction VI containing equal activity were pre-incubated at 37° in a standard reaction mixture lacking substrate, and periodically residual activity was measured at 25° with the DNA-gel assay. Both enzyme preparations lost activity exponentially, the wild type with a half-life of 15 min and the mutant with a half-life of 2 min. This finding further demonstrated that the mutant produces an altered DNase.

The purified enzymes (fraction VI) had the following properties characteristic of endonuclease II (11): (*a*) They had similar chromatographic elution profiles (steps IV to VI). (*b*) They released alkylated DNA from a polyacrylamide gel at rates >20 times (mutant enzyme) and >50 times (wild-type enzyme) those for untreated DNA. (*c*) They released DNA fragments; when 10–25% of the total radioactivity was released from a DNA gel, 90% of that released was acid-insoluble. We conclude that endonuclease II is the major endonuclease activity we are measuring in crude extracts (Table 1), that all three mutants are defective for this enzyme, and that BW2002 produces an altered enzyme and, therefore, has an altered structural gene for endonuclease II.

One Mutant, Two Enzymes. We examined seven mutants of independent origin which were originally identified as being deficient in either endonuclease II or in exonuclease III. In Table 3, we now see that all are defective for both activities. This finding suggests that the two enzymes depend on a common gene. Our interpretations are based on the assumption that our enzyme assays are specific even when applied to crude extracts. This specificity is suggested by the following

TABLE 3. *Simultaneous alteration of endonuclease II and exonuclease III activities by mutation*

Strain	Genotype	Specific activity (% of wild type) Endonuclease II	Exonuclease III
AB1157	*xth*$^+$	(100)	(100)
BW2001	*xth-11**	12	11
AB3027	No designation	9	11
BW9091	*xth-1*	8	14
BW9095	*xth-5*	29	12
BW9098	*xth-8*	27	15
BW9059	*xth-9*	10	16
BW9101	Δ(*pncA–xth*)	15	19

Growth of cells and assays of sonicates were performed at 37°. AB3027 is an $MeSO_2OMe$-sensitive strain discovered by S. Ljungquist and T. Lindahl (personal communication) to be deficient in endonuclease II. Strains of the BW9000 series were isolated as exonuclease III mutants (7). BW9101 (to be separately described) has a spontaneous deletion of unknown extent affecting the *pncA* gene and a nearby *xth* locus.

* Previously designated *ntw*$^-$ (endonuclease II-deficient) (5), but now found to be indistinguishable from *xth*$^-$ mutations.

observations: (*a*) The low levels of residual activity in some of our mutants (Table 3) indicate relatively little interference by enzymes other than those affected by our mutations. (*b*) The identities of mutant enzymes detected by these assays were confirmed by purification (this paper and ref. 7). (*c*) Exonuclease III mutants, detected by DNase assays, had only 1–8% normal levels of DNA-3′-phosphatase, a unique activity of this enzyme (7). (*d*) Measurement of endonuclease II was little affected by exonuclease III because glucosylated T4 DNA is resistant to the latter (12); in a typical assay, less than 10% of the DNA released from a gel by wild-type extracts was acid-soluble. (*e*) Conversely, endonuclease II should little affect the measurement of exonuclease III either by releasing acid-soluble fragments or by increasing the concentration of substrate (chain termini) because nonalkylated DNA is a poor substrate for endonuclease II (4) and because exonuclease III is 75% saturated with substrate under our assay conditions (C. Milcarek and B. Weiss, unpublished results).

TABLE 4. *Simultaneous alterations of temperature coefficients by mutation: evidence for a common structural gene for endonuclease II and exonuclease III*

Strain	Genotype	Temperature coefficient, $Q_{25°}^{42°}$ Endonuclease II	Exonuclease III
AB1157	*xth*$^+$	2.0	2.2
BW2002	*xth-12**	1.0	0.8
BW2003	*xth-13**	0.8	1.1
BW9093	*xthA3*	0.9	0.6
BW9094	*xth-4*	0.8	0.4
BW9097	*xth-7*	0.9	0.6

Cells were grown at 25° and sonicates were assayed at 42° and at 25°. $Q_{25°}^{42°}$ is the ratio of an enzyme activity at 42° to that at 25°. At 25°, all mutants had ≥70% of wild-type enzyme activities, except BW9094, which had 35% of wild-type endonuclease II activity.

* Previously designated as *ntw*$^-$ (5).

Temperature-Sensitive Mutations. Table 4 shows that mutants having an altered temperature coefficient for endonuclease II activity are also temperature-sensitive for exonuclease III. Strains BW2002 and BW2003 were originally isolated as endonuclease II mutants, whereas the others were originally isolated as exonuclease III mutants. A temperature sensitive exonuclease III was in fact purified from BW9093 (7). The results indicate that both enzymes depend on a common structural gene and are, therefore, physically associated. This association was further implied by co-purification. We found that a highly purified preparation of exonuclease III (fraction VI, ref. 7) contained endonuclease II activity of equal purity. Conversely, during the purification of endonuclease II, others have noted the co-purification of an exonuclease activity (see *Discussion*). Our own preparations of endonuclease II (Table 2), however, were consumed for other studies before we could confirm this finding.

Biological Traits. Our strains were tested at 42° except for phage growth, which was performed at 37°. The three new endonuclease-II-deficient mutants were sensitive to bacteriophages T4 and T7, had no apparent defect in conjugational or transductional recombination, and had no apparent alteration of spontaneous mutation frequency for several genetic markers. BW2001 had only a slightly increased sensitivity to ultraviolet and γ-irradiation (Fig. 1). Although not shown in Fig. 1, BW2002 had an extraordinary sensitivity to mitomycin C (10^{-6} survival at 1 μg/ml), but this property was probably the result of a secondary mutation, because it was not cotransducible with the enzymatic defects. Of the three mutants, only BW2001, the one with the least residual enzyme activity, demonstrated major biological abnormalities (Fig. 1 and Table 5). It had a prolonged generation time in nutrient broth, an unusual sensitivity to the alkylating agents $MeSO_2OMe$ and mitomycin C, and a poor plating efficiency for bacteriophage λ.

Phenotypic Revertants. Strain BW2007, a faster-growing revertant of BW2001, was isolated from a larger colony arising spontaneously after multiple serial transfers of a broth culture. BW2021, an $MeSO_2OMe$-resistant revertant of BW2001, was isolated as a survivor from an agar plate containing a central droplet of $MeSO_2OMe$. The data of Table 5 show that all the described phenotypic abnormalities of strain BW2001 can revert simultaneously; therefore, these defects are most likely due to a single mutation, *xth-11*, rather than multiple independent mutations. Strain BW2007 did not have a detectable increase in exonuclease III activity, but then its increase in endonuclease II was relatively small.

Preliminary Genetic Mapping. Phage P1 lysates of the mutants were used to transduce nearby auxotrophic markers, and the transductants were scored for *xth*$^-$ by assay of endonuclease II or exonuclease III activities. Transductants tested by both assays (*xth-9*, *xth-11*, and *xth-12*) were found to have co-inherited both enzymatic defects, supporting our assumption that they were caused by single mutations. The mutations of strains BW2001, BW2002, and BW2003 (*xth-11* to *xth-13*) were 2–9% cotransducible with the *aroD* gene and 17–36% cotransducible with *pncA*, whereas *aroD* was only

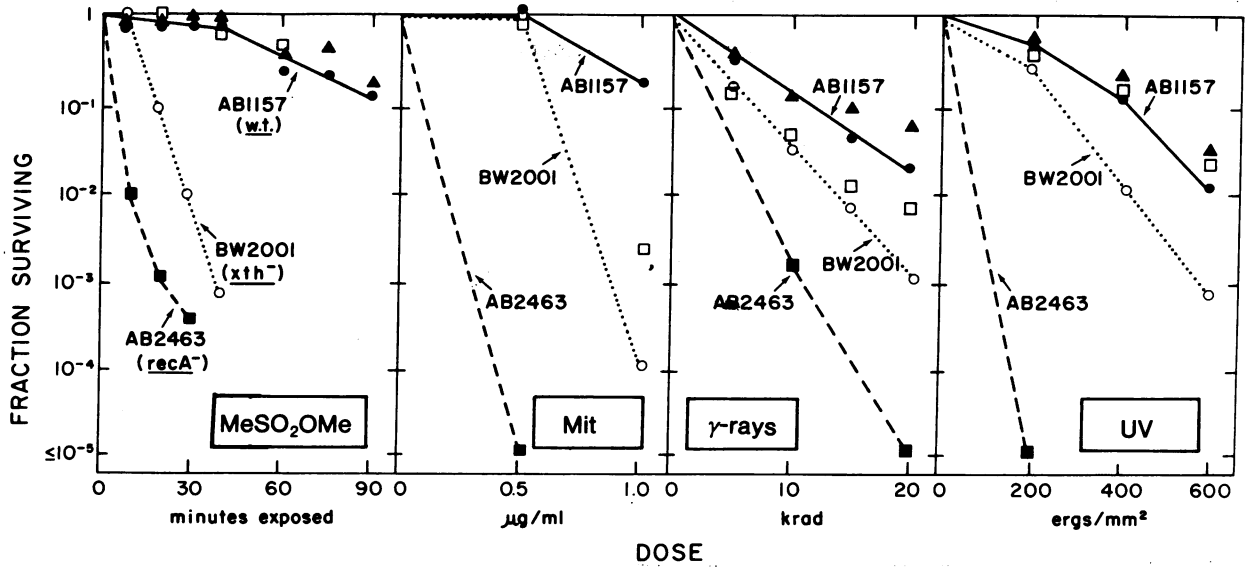

FIG. 1. Sensitivity of new endonuclease II mutants to alkylating agents and to irradiation. AB2463, a recombination-deficient *xth*⁺ strain, is sensitive to all agents and was included for comparison. Abbreviations: w.t., wild type; Mit, mitomycin C; UV, ultraviolet light. Symbols: ●——●, AB1157; O····O, BW 2001; ▲, BW2002; □, BW2003; ■– – –■, AB2463.

0.5% cotransducible with *pncA*. Therefore, the mutations are between the *aroD* and *pncA* genes at about 33 min on the chromosomal map of *E. coli* (13). The *xth-1* to *xth-9* and Δ(*pncA-xth*) mutations are located in or near the same region (ref. 14 and unpublished results).

DISCUSSION

The results indicated that in *E. coli* endonuclease II and exonuclease III activities are associated. Twelve independent mutations affected both activities and were located in the same region of the chromosome, and a phenotypic revertant of one recovered both enzyme activities. The association between endonuclease II and exonuclease III appears to be a physical one because five mutations affected a physical property (thermal stability) of both enzymatic activities. These activities may be associated in one of several ways: they may share the same active site; they may have different catalytic sites on the same polypeptide chain; they may each be catalyzed by part of a larger protein complex; or they may be activities of separate enzymes that are fortuitously co-purified and that contain a common polypeptide subunit. Our data cannot discriminate among these possibilities; the two enzymes may or may not be identical, and only physicochemical studies of highly purified preparations can resolve this question.

The co-purification of the two enzymes also suggested their physical association. Friedberg and Goldthwait (1) and Hadi *et al.* (11) found their endonuclease II preparations were "contaminated" by an exonuclease which, like exonuclease III, was relatively inactive on the glucosylated DNA of phage T4, although its inhibition by Zn^{++} was less than they expected for the latter enzyme. Their separation of the two activities by electrophoresis resulted in an 80–90% loss of endonuclease activity (11). Although isoelectric focusing produced several peaks of exonuclease-free endonuclease II in unstated yield, the recovered exonuclease activity coincided with a peak of endonuclease II activity (11). The possibility of separating these activities, whether by dissociation or proteolysis, might enable future reconstruction experiments with purified preparations or with extracts of mutants (i.e., *in vitro* complementation).

Excision-repair of lesions in DNA may be accomplished by the sequential action of an endonuclease, an exonuclease, a polymerase, and a ligase. The physical association of two or more such enzymes is of obvious physiological advantage, as evidenced by *E. coli* DNA polymerase I, a possible repair enzyme with combined exonuclease and polymerase activities (15). Similarly, an activity resembling that of *E. coli* exonuclease III accompanies an endonuclease II-like activity of *Hemophilus influenzae* during purification of the latter to apparent homogeneity, suggesting a general phenomenon (J. Gargiulo and B. Weiss, unpublished results).

TABLE 5. *Properties of strain BW2001 and its phenotypic revertants*

Property	Strain AB1157 (Parent)	BW2001 (Mutant)	BW2007 (Revertant)	BW2021 (Revertant)
Endonuclease II level (%)*	(100)	10	23	112
Exonuclease III level (%)*	(100)	11	11	62
E.o.p. of phage λ*vir*†	(1.0)	0.05	1.0	1.0
Generation time (min)	27	60	36	48
Mitomycin C resistance (% surviving 1 μg/ml)	55	0.018	56	46
$MeSO_2OMe$ resistance (% surviving 24 mM 40 min)	79	0.0012	0.5	42

* Specific activities of extracts were determined and recorded as in Table 3.

† E.o.p. (efficiency of plating) = the ratio of a phage titer obtained with a given bacterial strain to that obtained with AB1157 as indicator. The plaques obtained with BW2001 were not only reduced in number but were also about 20% of normal diameter. λ*vir* is a virulent mutant.

The unusual sensitivity of mutant BW2001 to alkylating agents is consistent with the hypothesis that endonuclease II functions in the excision-repair of alkylated DNA. This hypothesis is further supported by the work of S. Ljungquist and T. Lindahl (personal communication). They found that AB3027 (Table 3), an $MeSO_2OMe$-sensitive strain, is deficient in both endonuclease II and DNA polymerase I and that this double mutant is more sensitive to $MeSO_2OMe$ than either of the corresponding polymerase- or endonuclease-deficient single mutants.

Mutant BW2001 also had a reduced growth rate and a reduced ability to replicate phage λ, suggesting a possible role for the affected *xth* gene in normal DNA metabolism. It has been suggested (16) that endonuclease II may be important in the maintenance of cellular DNA, which may undergo depurination spontaneously. Such apurinic sites, which may also be produced by alkylating agents, are ambiguous templates and may lead to mutation. We are unable, however, to attribute the slow growth rate of strain BW2001 to the accumulation of spontaneous mutations; in preliminary experiments, its mutation rates for several traits are equal to or less than those of the wild type. Finally, we must point out that the mutation in strain BW2001 may not be in a structural gene for the enzymes; it may be in a control locus affecting other genes that are responsible for the mutant's biological properties.

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