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Escherichia coli Mutants Deficient in Deoxyuridine Triphosphatase

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Mutants deficient in deoxyuridine triphosphatase (dUTPase) were identified by enzyme assays of randomly chosen heavily mutagenized clones. Five mutants of independent origin were obtained. One mutant produced a thermolabile enzyme, and it was presumed to have a mutation in the structural gene for dUTPase, designated *dut*. The most deficient mutant had the following associated phenotypes: <1% of parental dUTPase activity, prolonged generation time, increased sensitivity to 5'-fluorodeoxyuridine, increased rate of spontaneous mutation, increased rate of recombination (hyper-Rec), an inhibition of growth in the presence of 2 mM uracil, and a decreased ability to support the growth of phage P1 (but not T4 or λ). This mutation also appeared to be incompatible with *pyrE* mutations. A revertant selected by its faster growth had regained dUTPase activity and lost its hyper-Rec phenotype. Many of the properties of the *dut* mutants are compatible with their presumed increased incorporation of uracil into DNA and the subsequent transient breakage of the DNA by excision repair.

In *Escherichia coli*, dUTP arises by two mechanisms: by the enzymatic deamination of dCTP (29) and by the reduction of UDP to dUDP that is then phosphorylated to dUTP (6). The first line of defense against the incorporation of dUTP into DNA is the enzyme deoxyuridine triphosphatase (dUTPase; dUTP nucleotidohydrolase [EC 3.6.1.23]). This enzyme, which is more active than any other deoxynucleoside triphosphatase in *E. coli* extracts, is highly specific for dUTP and hydrolyzes it to dUMP and PP_i (6, 12), thereby also producing most, if not all, of the dUMP needed for the synthesis de novo of thymidylate (4). If any dUTP escapes dUTPase and is incorporated into DNA, the uracil of dUMP residues may be excised from the DNA in several ways. Thus, a uracil-DNA glycosylase (19) may hydrolyze uracil from the DNA, leaving apyrimidinic sites that are attacked by certain endonucleases (15, 20, 32, 34), or endonuclease V (9) may directly recognize and attack uracil-containing DNA.

By isolating mutants for dUTPase (*dut* mutants) we hoped to study the biological consequences of the misincorporation of uracil into cellular DNA. In preliminary studies of our mutants, however, we could find no uracil in their DNA (S. J. Hochhauser and B. Weiss, Fed. Proc. 35:1492, 1976). We presumed that the excision repair systems might be so highly efficient that we should have to examine newly synthesized DNA to find any repair intermediates. We then found that our *dut* mutations were located near the *dnaS* or *sof* mutations of Konrad and Lehman (18). These latter mutants had been isolated on the basis of an unusually high recombination frequency (hyper-Rec phenotype), a probable consequence of their excessive fragmentation of newly synthesized DNA resulting in very short Okazaki fragments (Sof phenotype). Collaborative studies (31) confirmed the identity of the *dut* and *sof* (*dnaS*) genes. All *dut* and *sof* mutants were deficient in dUTPase and appeared to have the hyper-Rec and Sof phenotypes to a degree related to their enzyme deficiencies. Tye and Lehman (30a) studied *dut* mutants that also lacked uracil-DNA glycosylase or dCTP deaminase and confirmed the hypothesis that, in *dut* mutants, the fragmentation of nascent DNA results from the excision of misincorporated uracil.

In this work we shall describe the isolation of our *dut* mutants used in the above studies, the isolation of a revertant, the apparent incompatibility between *pyrE* and *dut* mutations, and additional traits associated with the tight *dut* mutations. We shall also provide further evidence for the relationship between dUTPase deficiency and the hyper-Rec phenotype.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this study are listed in Table 1. Gene symbols are

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TABLE 1. *Bacterial strains*

Strain	Genotype	Derived from:	Source
AB1157	*thi-1 argE3 leu-6 thr-1 proA2 his-4 rpsL31 mtl-1 xyl-15 ara-14 galK2 lacY1 tsx-33 supE44*		A. J. Clark
AT2538	*thi-1 pyrE60 argE3 leu-6 thr-1 proA2 his-4 rpsL31 mtl-1 xyl-15 ara-14 galK2 lacY1 supE44*?		CGSC[a]
BW192	KS468 *pyrE*$^+$	KS468	P1 transduction
BW3001 through BW3005	AB1157 *dut-1* through *dut-5*	AB1157	Mutation
BW3050	AT2538 *pyrE*$^+$	AT2538	P1 transduction
BW3051	AT2538 *pyrE*$^+$ *dut-1*	AT2538	P1 transduction
BW3060	*tfr-8*	JM15	P1 transduction
BW3061	*tfr-8 dut-1*	JM15	P1 transduction
BW3068	*tfr-8 dut-11*[b]	JM15	P1 transduction
BW3101	KS468 *pyrE*$^+$ *dut-1*	KS468	P1 transduction
BW3102	KS468 *pyrE*$^+$ *dut-2*	KS468	P1 transduction
CS5	HfrC *gltC10 metB1 relA1 tonA22* T2^r *spoT1*?		CGSC
JM15	*tfr-8 cysE50*		CGSC
KS468	*metB thi pyrE lacMS286*ϕ80dII*lacBK1* Str^r		I. R. Lehman (18)
RS5087	KS468 *dut-11*[b]	KS468	I. R. Lehman (18)

[a] CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.
[b] The *dut-11* mutation has previously been designated *dnaS1* (18) and *sof-1* (31).

those of Bachmann et al. (2).

Media. Nutrient media, minimal media, and supplements were as previously described (36). Phages λ and T4 were grown on tryptone medium (36), and P1 was grown on R medium (25). Lactose-tetrazolium and M9-glucose media were as described by Miller (25). M9 medium containing 0.2% vitamin-free Casamino Acids (Difco) was employed as a pyrimidine-free medium in some experiments. Lactose-minimal medium contained glucose-free lactose obtained from Sigma Chemical Co. or U.S. Biochemical Corp. (NRC grade).

Preparation of γ-^{32}P-labeled deoxynucleoside triphosphates. The method of Glynn and Chappell (10) for synthesizing [γ-^{32}P]ATP was modified, as suggested by Mitra et al. (26), by adding a 20-fold excess of the nucleotide to be labeled and by adding nucleoside diphosphate kinase. A preparation of lactic dehydrogenase from rabbit muscle (Sigma, type I, crude) served as a source of this enzyme (suggested by P. Englund). The nucleotides were purified by chromatography on diethylaminoethyl-Sephadex A-25 (Pharmacia Fine Chemicals, Inc.) with a linear gradient of 0.25 to 0.6 M triethylammonium bicarbonate (26). Purity was ascertained by thin-layer chromatography on polyethyleneimine cellulose sheets, with 1.6 M LiCl as solvent (30).

Other materials. dUMP and dUTP were purchased from P-L Biochemical Corp. 5'-Fluorodeoxyuridine (FUdR) was obtained from Sigma Chemical Co., and uracil was obtained from Calbiochem. Other nucleotides, nucleosides, and nucleic acid bases were obtained from Calbiochem and Schwarz/Mann. Carrier-free $^{32}P_i$ was purchased from New England Nuclear Corp. [5'-^{3}H]dUTP was obtained from Amersham/Searle and checked chromatographically before use. Plastic-backed sheets of polyethyleneimine cellulose for thin-layer chromatography were obtained from Brinkmann Instruments.

Mutagenesis. The multiple auxotroph AB1157 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and clones of the mutagenized cells were stored in 20% glycerol at −70°C in 96-well depression plates, as previously described (24, 35).

Mass assays for dUTPase. Assay A, a semiquantitative screening assay, was used for the initial identification of *dut* mutants and for the scoring of dUTPase deficiency in transduction experiments. It measured the hydrolysis of [γ-^{32}P]dUTP to yield $^{32}PP_i$, which was detected as Norit-nonadsorbable radioactive material. The assays were performed in microwell plates, using previously described techniques for bacterial growth, lysis, pipetting, mixing, centrifugation, and sampling (24, 35). Each clone to be assayed was grown in 50 μl of nutrient broth. For the initial mutant search, growth was at 25°C for 2 days; in subsequent experiments, it was for 1 day at 37 or 42°C. BW3001 and its derivatives usually required 2 days at 37°C to reach saturation. The cultures, in individual wells of a depression plate, were pelleted by centrifugation and suspended in 50 μl of a solution containing lysozyme (0.1 mg/ml) and 5 mM trisodium ethylenediaminetetraacetic acid in 50 mM tris(hydroxymethyl)-aminomethane (Tris)-maleate buffer (pH 6.5). After 5 min at 25°C, the mixtures were subjected to three cycles of freezing at −70°C and thawing at 25°C. Before the final thawing, the plates could be stored at −70°C for several weeks without noticeable loss of activity. To the lysed cells was added 50 μl of 0.4 mM [γ-^{32}P]dUTP (1.3×10^6 to 3.8×10^6 cpm/μmol) in 50 mM Tris-maleate buffer (pH 6.5). After 30 min of incubation at 37 or 42°C, 50 μl of a Norit suspension was added. (To prepare the Norit suspension, fines were first removed by repeated decantation; the Norit suspension was then adjusted to 20% packed volume in 0.3 N HCl–6.25 mM sodium PP_i–6.25 mM KH_2PO_4–1.25 mg of bovine plasma albumin per ml and degassed with an aspirator). Vigorous mixing for 5 min was followed by centrifugation for 15 min at $3,000 \times g$. Ten-microliter samples of each supernatant were transferred to Norit-impregnated filter paper (Schleicher and Schuell no. 508) with a manifold of 12 glass capillary tubes (35) fitted with a manifold of wire

plungers (Drummond Scientific Co.). The paper was dried and placed against X-ray film (Kodak No-Screen) for enough time for an average of 6×10^5 disintegrations from samples with wild-type activity. Relative enzyme activities were judged from the intensities of the spots on the developed radioautograph (24, 35). Many of the spots had very dark centers that were ignored; they represented unadsorbed nucleotides and unsedimented Norit in the supernatants. Assay conditions were such that *dut*$^+$ cells hydrolyzed 30 to 40% of the substrate.

Assay B was a more quantitative mass assay used for the accurate scoring of transductants with leaky *dut* mutations. To lysates prepared as in assay A was added 50 μl each of a reaction mixture containing 0.4 mM [5′-^{3}H]dUTP (10^7 cpm/μmol) in 50 mM sodium morpholinopropane sulfonate (MOPS) buffer (pH 6.5). After 30 min at 37°C, the plates were transferred to an ice bath, and 20 μl of 50% ethanol was added to each well. After mixing and centrifugation (15 min at $3,000 \times g$), 25-μl samples were applied to polyethyleneimine cellulose sheets, and the chromatograms were developed 5 cm with 2 M sodium formate buffer (pH 3.45) (30). Radioactive dUMP was measured in the top centimeter; dUTP remained at the origin.

Quantitative dUTPase assays. Cells were grown in nutrient broth to 5×10^8/ml and sonically treated as previously described (24), except that 50 mM Tris-maleate buffer (pH 6.5) was used for washing and sonic treatment. The sonically treated material could be stored at −20°C for 1 year with <10% loss of activity. For assay C (similar to assay A), the reaction mixture (0.3 ml) contained 30 nmol of [γ-^{32}P]dUTP (10^4 to 10^6 cpm total), 15 μmol of Tris-maleate buffer (pH 6.5), and 0.1 to 0.5 unit of enzyme (e.g., 10 μl of an exponential- or 5 μl of a stationary-phase cell extract). Incubation was for 30 min at 37°C unless otherwise noted. The tubes were then transferred to an ice bath, and the following were added in order: 0.2 ml of 0.4 N HCl, 0.1 ml of bovine plasma albumin (5 mg/ml) in 0.025 M sodium PP_i–0.025 M potassium phosphate buffer (pH 7.0), and 0.2 ml of a 20% (packed volume) Norit suspension. (The Norit suspension had first been prepared by removal of fines through repeated decantation, followed by degassing in vacuo.) The tubes were kept on ice for 5 to 10 min and shaken intermittently. After centrifugation for 5 min at $12,000 \times g$, radioactivity was determined in 0.4 ml of the supernatant. In the absence of enzyme, 1 to 5% of the radioactivity in the reaction mixture was Norit nonadsorbable. Values were proportional to the enzyme concentration in the range given (up to 50 to 60% hydrolysis). One unit of enzyme, as defined by Greenberg (11), is that which catalyzes the hydrolysis of 1 nmol of dUTP per min at the indicated temperature.

Assay D, similar to assay B, measured the production of dUMP from dUTP. The cultures were prepared and sonically treated as for assay C, except that 50 mM MOPS buffer (pH 6.5) was used in place of Tris-maleate. The reaction mixture (50 μl) contained 6 nmol of [5-^{3}H]dUTP (5×10^4 cpm total) in 30 mM MOPS buffer (pH 6.5). To this was added 10 μl of enzyme (0.004 to 0.02 unit) diluted in 50 mM MOPS buffer (pH 6.5). Incubation was for 30 min at 37°C unless otherwise noted, and the tubes were then transferred to an ice bath. To each was added 10 μl of a carrier solution containing 1 to 2 mM each unlabeled dUMP and dUTP. A 20-μl sample of each mixture was applied to a polyethyleneimine cellulose thin-layer sheet and developed 10 cm with 1.0 M LiCl. After the chromatogram was dried, each dUMP spot was visualized under UV light, and its radioactivity was measured without prior elution. Values were proportional to the enzyme concentration in the range described. Units were defined as in assay C.

Hydrolysis of deoxynucleoside triphosphates by crude extracts. Cells were grown at 37°C and sonically treated as described for assay C. Reaction mixtures (2.5 ml) contained 125 μmol of Tris-maleate buffer (pH 6.5), 250 nmol of [γ-^{32}P]dUTP, -UTP, or -dTTP (0.5×10^6 to 1.0×10^6 cpm total), and 0.1 ml of cell extract. Incubation was at 37°C. Samples (0.2 ml) were removed periodically and added to 0.1 ml of cold 0.6 N HCl. Norit-nonadsorbable radioactivity was determined as for assay C.

Heat inactivation of dUTPase activity. Cell extracts were prepared as for assay D, diluted in 50 mM MOPS buffer (pH 6.5) to protein concentrations of 78 μg/ml (BW192) or 310 μg/ml (BW3102), and incubated at 42°C. Samples (10 μl) were removed periodically, and dUTPase activity was determined by assay D at 30°C in prewarmed reaction mixtures.

Scoring the hyper-Rec phenotype. Strains bearing the *lac* diploid markers (*lacMS286*φ80dII-*lacBK1*) are Lac$^-$ but can become Lac$^+$ by a recombination of their two partial *lac* regions (17). Colonies of the strains to be tested were grown to saturation in individual wells of a microwell plate and transferred via a 48-pronged inoculator (35) to a lactose-tetrazolium agar plate. After 2 to 3 days of incubation at 37°C, the relative number of white (Lac$^+$) papillae in each red (Lac$^-$) cell patch was noted. *dut-1* and *dut-11* strains had about two to five times as many papillae as did *dut*$^+$ cells. Questionable results were checked by quantitative assays of recombination frequency.

Measurement of recombination frequency. For measurement of recombination frequency (38), the *lac* diploid strains were grown to a density of 1.5×10^8 cells per ml at 37°C in nutrient broth without added glucose. Then 0.1 ml of each culture was plated on a lactose minimal medium in a soft agar overlay. After incubation at 37°C for 2 to 3 days, colonies (Lac$^+$ recombinants) were counted and compared with the total number of viable cells determined by plating dilutions of the cultures on nutrient media.

Mutation rates. The reversion of *argE3* mutations was determined as previously described (24), except that growth was at 37°C. Mutations to valine resistance were similarly determined, except that the cultures were washed with minimal medium and plated on minimal medium containing 160 μg of L-valine per ml.

Isolation of faster-growing derivatives of BW3001. BW3001 was grown overnight in nutrient broth at 37°C, diluted 100-fold into fresh broth, and grown again to saturation. After 10 such serial passages, the cells were streaked onto nutrient agar, and the largest colonies were picked for further study. Their amino acid requirements were those of BW3001.

Sensitivity to uracil and FUdR. Cultures were grown overnight in nutrient broth at 37°C, and the cells were centrifuged and suspended three times in 50

mM potassium phosphate buffer (pH 7.4). They were diluted in the same buffer and spread on minimal media containing the stated concentrations of either uracil or FUdR. After incubation for 2 days at 37°C, plates containing at least 100 colonies were counted.

Other methods. The following were determined by published methods: UV sensitivity (13), protein concentrations (21), and radioactivity measurements by liquid scintillation counting (24). Toluenized cells were prepared as described by Moses (27), except that 50 mM glycine-NaOH buffer (pH 9.4) was used, and toluenization was carried out for 15 min at 37°C. These conditions were found to give higher dUTPase activity, with no apparent cell lysis, than the gentler conditions generally employed for studying DNA replication.

RESULTS

dUTPase activity in cell-free extracts. Preliminary experiments with sonic extracts of AB1157 (*dut*$^+$) indicated that this *E. coli* K-12 strain possessed a dUTPase activity similar to those previously reported in *E. coli* B (6) and *E. coli* R2 (12). The activity had a pH optimum of 6.5 and an apparent K_m of 9.9 μM. The activity was not stimulated by the addition of Mg^{2+}, but its inhibition by ethylenediaminetetraacetic acid (50% at 1 mM) was reversed by 10 mM Mg^{2+}. The release of radioactivity in a Norit-nonadsorbable form (i.e., as P_i or PP_i) from either [γ-^{32}P]dTTP or [γ-^{32}P]UTP occurred at <5% of the rate of [γ-^{32}P]dUTP in crude extracts under standard assay conditions. Therefore, there should be little interference by nonspecific nucleoside triphosphatases in the assay to be used for screening for dUTPase mutants.

Isolation of the mutants. Strain AB1157, a multiple auxotroph, was heavily mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and random clones were assayed for dUTPase activity. The cells were assayed at 42°C, but grown at 25°C to permit the propagation and identification of possible conditional lethal mutants. Among 7,500 randomly assayed clones, five mutants of independent origin were identified. None was temperature sensitive for growth. Their enzyme levels are shown in Table 2.

The most deficient mutant was BW3001 (*dut-1*). Its dUTPase activity was reduced to a level comparable with the rate of breakdown of dTTP and UTP (Fig. 1), and we do not know, therefore, how much of its residual dUTPase activity was due to nonspecific triphosphatases. These assays, moreover, did not distinguish between the release of $^{32}PP_i$ and that of $^{32}P_i$. By a more specific chromatographic assay (assay D) that measured the production of dUMP from dUTP, BW3001 was found to have <1% of wild-type dUTPase activity at 37°C. The rates of hydrolysis of UTP and dTTP (Fig. 1) were not affected by this mutation. This finding was compatible with the high degree of specificity reported for partially purified dUTPase obtained from other *E. coli* strains (6, 12, 33).

TABLE 2. *dUTPase levels of mutants*[a]

Strain	Allele	dUTPase activity (% of wild type)[b] at: 25°C	42°C	Temp coefficient, Q_{25}^{42}[c]
AB1157	*dut*$^+$	(100)	(100)	1.60
BW3001	*dut-1*	5.1	0.8	—[d]
BW3002	*dut-2*	44.8	19.2	0.69
BW3003	*dut-3*	8.2	11.1	2.19
BW3004	*dut-4*	11.5	7.7	1.08
BW3005	*dut-5*	24.0	28.3	1.89

[a] Cells were grown at 25°C, harvested during logarithmic growth, and assayed for their ability to release Norit-nonadsorbable radioactivity from [γ-^{32}P]dUTP (assay A).

[b] Wild-type (AB1157) dUTPase levels were 7.8 and 12.6 units per mg of protein at 25 and 42°C, respectively.

[c] Q_{25}^{42} = ratio of the activity at 42 to that at 25°C.

[d] dUTPase levels were too low for calculation of a meaningful ratio.

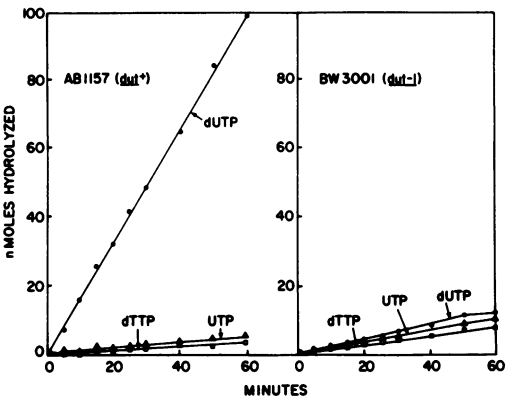

FIG. 1. *Specific hydrolysis of dUTP by extracts of E. coli K-12. Hydrolysis of* γ-^{32}*P-labeled triphosphates was measured by the release of Norit-nonadsorbable* ^{32}*P. The AB1157 reaction mixtures contained less protein (42 μg/ml) than did the BW3001 mixtures (65 μg/ml).*

We explored the possibility that the mutant enzymes were active in vivo but were inactivated by disruption of the cells. Instead of sonically treating or lysing the cells, we treated them with toluene to render them permeable to the labeled substrate while leaving their membranes intact. Levels of dUTPase in the toluenized mutants (data not shown) were similar to those in the sonically treated mutants (Table 2). We concluded that the mutant enzymes were inactive in vivo as well as in vitro. Confirmation was

supplied by independent studies (31) showing that the mutants had an abnormality during growth (excessive fragmentation of new DNA) that paralleled their dUTPase deficiencies.

Mutation in a structural gene for dUTPase. When extracts of each of the mutants were mixed with those of wild-type cells and assayed, the results were additive, suggesting that the mutants were not producing an inhibitor in excess, but were defective in enzyme production. Three mutants of independent origin lacked about 90% or more of dUTPase activity (Table 2), suggesting that there is a single major dUTPase in wild-type cells. In at least one mutant, BW3002 (*dut-2*), this protein was clearly altered, having become thermolabile. The dUTPase in this strain was less active at 42°C than at 25°C (Table 2). The instability of the mutant enzyme was demonstrated more strikingly in an experiment (Fig. 2) in which an extract of a *dut-2* transductant was incubated at 42°C in the absence of added substrate. The *dut-2* enzyme was inactivated with a half-life of 20 min, whereas that from the congenic *dut*$^+$ strain increased in activity during the incubation. Two other separate observations were consistent with the instability of this mutant enzyme. (i) BW3002 (*dut-2*) grown to saturation in broth at either 30 or 42°C had <2% of the wild-type activity when assayed at either temperature; the enzyme was therefore irreversibly inactivated by growth into stationary phase. (ii) The altered enzyme could not be recovered in even the earliest of purification steps.

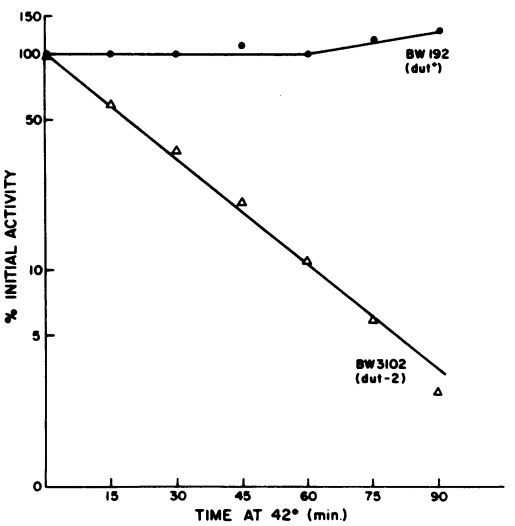

FIG. 2. *Thermal instability of BW3102 (dut-2) dUTPase activity. Duplicate cell extracts were preincubated at 42°C in the absence of substrate and then assayed at 30°C. Each point represents the average of three experiments. At zero time, average specific activities in units per milligram of protein were 15.6 for BW192 and 3.7 for BW3102.*

The unique specificity of the crude dUTPase and its almost complete absence in nonselected mutants after one-step mutagenesis suggested that there was only one major dUTPase in *E. coli* and obviated the need for purifying the altered *dut-2* enzyme for the purpose of identification. The *dut-2* mutation results in an altered protein and is therefore within a structural gene for dUTPase.

Other *dut* mutants. In addition to our five mutations (*dut-1* to *dut-5*), we studied three others (*dut-11* to *dut-13*) that were isolated by Konrad and Lehman (18). These latter mutations were previously referred to as *dnaS1, -2,* and *-3* (18), or as *sof-1, -2,* and *-3* (31), respectively; the corresponding mutants possessed 5, 7, and 12% of wild-type dUTPase activity at 37°C. In a previous study (31), all eight mutants were shown to have the Sof (short Okazaki fragment) phenotype to a degree commensurate with their dUTPase deficiencies. In the studies described below, we found marked biological abnormalities only for strains bearing the *dut-1* and *dut-11* alleles, i.e., the most deficient strains. Data are therefore presented only for these strains. In these experiments, *dut-1* and *dut-11* transductants, rather than the original heavily mutagenized parents, were studied.

Apparent *pyrE-dut* incompatibility. In transductions mediated by phage P1, seven of the mutations were 80 to 90% linked with the *pyrE* locus at 81 min on the genetic map of *E. coli* (31): the *dut-12* mutation, scored only by the somewhat ambiguous assay for the hyper-Rec phenotype, appeared to be 98% cotransducible with *pyrE*. Two-factor linkage analysis (18) suggested the clockwise gene sequence *cysE-dut-pyrE,* but the relative order of *dut* and *pyrE* was uncertain. We attempted to determine that order by transductional three-point linkage analysis. Multiple crosses, including one set of reciprocal crosses, were performed between *dut-1 pyrE*$^+$ and *dut*$^+$ *pyrE* parents. In each cross, selection was for one of the following three markers flanking the *pyrE-dut* region: *cysE*$^+$, counterclockwise of and 11% cotransducible with *pyrE* (18); *gltC10,* clockwise of and 18% cotransdicible with *pyrE* (23); or tetracycline resistance, from a Tn*10* element clockwise of and 70% cotransducible with *pyrE* (see below). A total of 405 recombinants were examined, and the expected linkages between each pair of selected and nonselected markers were seen. Although 17 *pyrE*$^+$ *dut*$^+$ recombinants were found (1 to 7 in each of five crosses), not one *pyrE dut-1* strain was isolated. In reciprocal crosses, some of the *pyrE*$^+$ *dut*$^+$ recombinants must necessarily have

arisen from rare quadruple crossovers, and *pyrE dut* strains should have been produced by more frequent double crossovers, but whereas the former were always found, the latter never were. The systematic exclusion of one class of recombinants precluded the determination of the gene order by three-point linkage analysis, and the data are therefore not shown. There is no apparent reason why the cell should not tolerate a combined deficiency of orotidylate pyrophosphorylase (the *pyrE* product) and dUTPase.

We attempted to determine the relative position of *pyrE* and *dut* in a three-point cross in which we used the leaky *dut-4* allele, and we did obtain *pyrE dut-4* recombinants. Unfortunately, accurate scoring of the *dut-4* genotype required quantitative assays, thereby limiting the number of transductants that we could feasibly examine, and crossovers did not occur between *pyrE* and *dut* with sufficient frequency to provide reliable data.

Hyper-Rec phenotype. *dut* mutants displayed a higher-than-normal recombination proficiency, or hyper-Rec phenotype (18, 31). It was most prominent in strains containing *dut-1* or *dut-11,* the tightest mutations (31). This property was detected by the semiquantitative observation of Lac^+ recombinants arising from a *lac* diploid strain containing a deletion within each copy of the *lac* operon; such recombinants produced white papillae within red Lac^- colonies on lactose-tetrazolium medium (17). Actual measurements (Table 3) revealed that broth cultures of *dut-1* and *dut-11* strains contained 4 to 13 times as many Lac^+ recombinants as did their dut^+ counterparts.

As previously reported (31), an analysis of 184 $pyrE^+$ transductants obtained in crosses with *dut-1* donors showed a one-to-one correspondence between the inheritance of dUTPase deficiency and the hyper-Rec phenotype. As further evidence for the relationship between these traits, we examined AB1157, the dut^+ parent for the mutants *dut-1* to *dut-5.* In the cross P1(AB1157) × KS468, none of 88 $pyrE^+$ transductants tested was hyper-Rec. Therefore, mutations to dUTPase deficiency and to hyper-Rec must have occurred simultaneously in AB1157, just as they did in the parent for the *dut-11* to *dut-13* mutations.

Uracil sensitivity. The growth of some *dut-1* strains on solid media was inhibited by high concentrations of uracil. The cells had about the same survival whether plated in stationary or exponential phase. The most sensitive strain was BW3061 (Fig. 3). It had a plating efficiency of 4 × 10^{-5} in the presence of 2 mM uracil, a concentration that is 10 times that normally supplied to uracil-requiring (Pyr^-) mutants. Three survivors were picked and found to be uracil resistant (plating efficiency of 0.54 to 0.77 in 2 mM uracil) while remaining dUTPase deficient.

TABLE 3. *Recombination frequencies in dUTPase mutants*

Strain	Allele	lac^+ recombinants per 10^5 cells
KS468	dut^+	3.1
BW192	dut^+	2.9
BW3101	*dut-1*	13
RS5087	*dut-11*	37

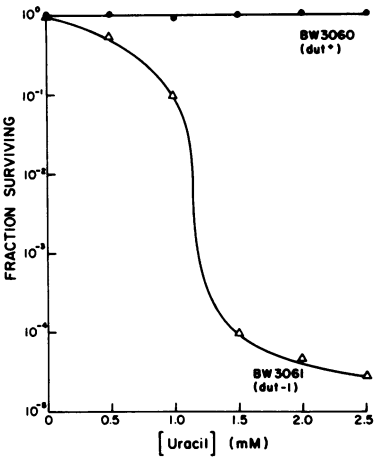

FIG. 3. *Uracil sensitivity of a dut-1 strain. Cells were plated on solid media containing the indicated concentration of uracil.*

The degree of uracil sensitivity varied unpredictably with the genetic background of the *dut-1* strains. Thus, BW3101 had a plating efficiency of 1.0 × 10^{-2} at 2 mM, whereas BW3051 appeared to be uracil resistant. Strain BW3068 (*dut-11* analog of BW3061) had a plating efficiency of 0.38 at 2 mM.

The effect of uracil was less striking in liquid medium. BW3061 was capable of growth in a liquid minimal medium supplemented with 1.8 mM uracil, but its doubling time at 37°C was 118 min in the presence of uracil and 80 min in its absence.

The effect of uracil on strain BW3061 was highly specific. Little or no effect (plating efficiency, >0.5) was found for cytosine, orotate, uridine, deoxyuridine, thymine, or deoxythymidine at 2 mM concentrations. The addition of thymine or deoxythymidine (2 mM) did not alter the effect of uracil on strain BW3061.

Sensitivity to FUdR. When grown in patches on agar media containing various levels of FUdR, *dut* mutants displayed sensitivities to FUdR that appeared to be commensurate with their enzyme deficiencies. This observation of

FUdR sensitivity was confirmed by a survival curve (Fig. 4) for the *dut-1* transductant BW3051. Although this strain was not sensitive to uracil (see above), it had an increased sensitivity to FUdR when compared with its congenic *dut*$^+$ parent. Whereas BW3051 was affected at the lowest concentration of FUdR tested (20 μM), its *dut*$^+$ counterpart had a threshold of about 60 μM, after which its viability declined (Fig. 4). It appears, therefore, that the *dut*$^+$ cells have some mechanism for preventing low levels of FUdR-induced damage and that this mechanism is overtaxed in *dut* cells.

Increased mutation frequency. Mutation frequency was measured by two tests: (i) reversion of *argE3* (ochre) strains to Arg$^+$ and (ii) forward mutations to valine resistance. The latter provided a rather general measure of mutation frequency because such mutations encompass alterations in a number of genes affecting the feedback regulation of isoleucine-valine biosynthesis (1). The results (Table 4) indicated significantly enhanced mutation rates for valine resistance but only a slight increase for *argE3* reversion. Therefore, certain types of mutation may be favored in *dut* mutants.

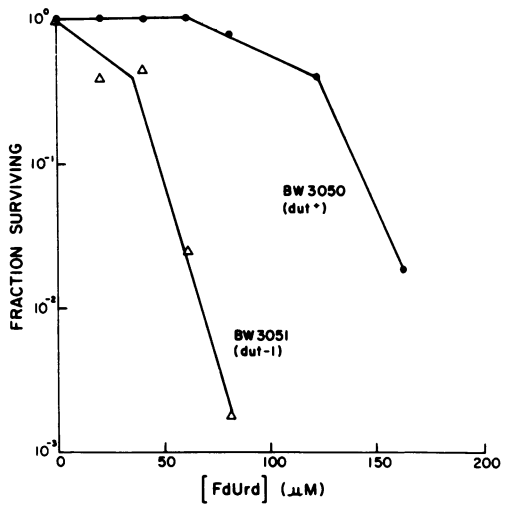

FIG. 4. *FUdR sensitivity of a dut-1 strain. Cells were plated on solid media containing the indicated concentrations of FUdR.*

Growth defect. Although the other mutants grew as well as the parental strain, the most deficient mutant, BW3001 (*dut-1*), had impaired growth (Table 5). We have transduced the *dut-1* mutation into many different genetic backgrounds and have observed that poor growth is a characteristic of all *dut-1* strains. The addition of thymine (125 mg/ml) or deoxythymidine (250 mg/ml) to pyrimidine-free medium (M9 plus 0.2% vitamin-free Casamino Acids) failed to stimulate their growth. These strains also exhibited a faster-than-normal loss of viability during storage in 50% glycerol at −20°C.

Isolation of a revertant. Two faster-growing derivatives of BW3001 (*dut-1*) were isolated by selection through growth in serially transferred liquid cultures. One had recovered dUTPase activity, and one had not (Table 5). Phage P1 lysates of the *dut*$^+$ revertant strain BW3031 were used to transduce the hyper-Rec indicator strain KS468 to *pyrE*$^+$. None of 70 transductants studied was hyper-Rec. Therefore, dUTPase deficiency, slow growth rate, and hyper-Rec phenotype reverted together, indicating that they were all due to the same mutation in BW3001.

Other biological traits. The growth of bacteriophage P1*virA* on *dut-1* and *dut-11* strains was impaired, but the *dut-2* to *dut-5* mutations had no apparent effect. The efficiency of plating of P1*virA* was 0.21 to 0.53 for the several *dut-1* strains and 0.53 for the one *dut-11* strain that were tested. T4 and λ phages grow normally on *dut* mutants; T4, however, induces a dUTPase activity (33), whereas λ does not (S. Hochhauser, unpublished data). The plating efficiency and burst size of φX174 is also reduced on *dut-1* hosts (A. Taylor and B. Weiss, unpublished data). The *dut-1* mutation did not affect survival after exposure to UV light at doses of up to 30 J/m^2. (Strain BW3001 [*dut-1*], however, had a mutation to UV sensitivity that was not linked to *dut* in P1 transduction experiments.)

General methods for the genetic transfer of *dut* markers. The preferred method for gene

TABLE 4. *Spontaneous mutation rates of dut-1 mutants*

Strain	*dut* allele	Marker selected	Frequency (mutants per cell)	*dut-1*/*dut*$^+$ [a]
BW3050	*dut*$^+$	*argE3* reversion	2.5×10^{-8}	1.5
BW3051	*dut-1*		3.7×10^{-8}	
BW3050	*dut*$^+$	Valine resistance	8.5×10^{-7}	5.2
BW3051	*dut-1*		4.4×10^{-6}	
BW3060	*dut*$^+$	Valine resistance	8.7×10^{-6}	14.9
BW3061	*dut-1*		1.3×10^{-4}	

[a] Ratio of the spontaneous mutation frequency of the *dut-1* mutant to that of the corresponding congenic *dut*$^+$ strain in each experiment.

TABLE 5. *Faster-growing derivatives of BW3001 (dut-1)*

Strain[a]	dUTPase activity[b] (% of wild type)	Doubling time[c] (min)
AB1157	(100)	29
BW3001	2	53
BW3021	2	41
BW3031	93	36

[a] BW3001 is a *dut* mutant of AB1157. BW3021 and BW3031 are spontaneous faster-growing derivatives of BW3001.

[b] dUTPase activity was assayed at 37°C.

[c] Generation times were determined in nutrient broth at 42°C.

transfer is P1 transduction because the transductant is >98% congenic with the recipient parent, and the latter can then serve as a control in biological experiments. Unfortunately, there are no satisfactory techniques for the direct selection of *dut* markers in transductants. Therefore, we introduced into *dut* mutants markers that are easily selectable in wild-type genetic backgrounds and that are closely linked to *dut*. The markers were *gltC10* and Tn*10*. *gltC10* (formerly *gltC5*) specifies the constitutive overproduction of glutamate permease and enables the cell to grow on glutamate as the sole carbon source (23). It was introduced into *dut* strains by P1 transduction with strain CS5 as the donor, and it was 20% cotransducible with *pyrE* and *dut*. Tn*10*, a translocation element specifying tetracycline resistance (16), was introduced into multiple loci in *E. coli* by transduction via a nonintegrating λ phage (λ55*b*221*c*I857*Oam*29-Tn*10*) obtained from N. Kleckner. Tet^r transductants were pooled, and a P1 lysate was prepared from them. It was used to transduce a *pyrE* strain to Pyr$^+$ Tet^r with simultaneous selection for both markers; recombinants, therefore, had Tn*10* insertions near *pyrE*. Three-factor crosses indicated that in one such transductant, Tn*10* was clockwise of and 70% cotransducible with both *pyrE* and *dut*. Tn*10 dut* strains were then constructed by P1 transduction.

When *gltC10 dut* strains were used as transductional donors, selection was on minimal medium containing 0.5% sodium L-glutamate. After incubation for 3 to 4 days at 37°C, transductants were picked and restreaked to single colonies on nutrient medium, rather than on selective medium, to save time. When Tn*10 dut* strains were used as donors, transductions were carried out in the usual way, except that after adsorption of phages, the cells were centrifuged, resuspended in nutrient broth containing 20 mM sodium citrate (to prevent reinfection), and aerated for 1 h at 37°C to permit gene expression. Selection was on nutrient agar to which tetracycline powder (25 mg/ml) was added before pouring.

If desired, initial screening for coinheritance of *dut* may be accomplished in one or more of the following ways: (i) dUTPase assays in microwell plates (assays A or B), (ii) hyper-Rec assays (in *lac* diploids only), or (iii) patch tests for uracil sensitivity (*dut-1* strains). For this last test, clones are grown in nutrient broth in microwell plates and transferred by means of a multipronged inoculator to the surface of an agar plate (tryptone or minimal agar) containing 2 mM uracil. Growth in patches is scored after 8 to 18 h at 37°C and compared with that of known *dut-1* and *dut*$^+$ strains. The *dut* genotype should always be confirmed by quantitative dUTPase assay, however, because that method is the least susceptible to error.

DISCUSSION

Many of the phenotypic traits of *dut* mutants can be explained by the transient incorporation of uracil into their DNA. A growing body of evidence (22, 30a, 34) suggests that the major enzymes involved in the subsequent excision repair are uracil-DNA glycosylase, exonuclease III (containing the major endonuclease activity directed against apurinic/apyrimidinic sites), DNA polymerase I, and DNA ligase. Thus, e.g., the double mutants *dut polA* and *dut lig* are each less viable than the corresponding single mutants (30a), and *dut xth* (exonuclease III) double mutants display filamentous growth (A. Taylor and B. Weiss, unpublished data). In *dut* mutants, newly synthesized (pulse-labeled) DNA strands are broken during the repair process, producing very short Okazaki fragments (Sof phenotype). Recombination, which involves breakage and joining of DNA strands, is probably increased by this fragmentation, resulting in the hyper-Rec phenotype. The competition between repair and replication for various substrates and enzymes might be responsible for the slow growth rate of *dut-1* strains and, in part, for their reduced yields of phages P1 and φX174. The increased frequency of spontaneous mutations in *dut* mutants might be explained by the diversion of enzymes from the repair of other lesions, such as base mismatches resulting either from errors in replication or from the spontaneous deamination of DNA cytosine. Alternatively, the fragmentation of DNA that accompanies uracil excision might lead to the induction of an error-prone repair system (37), a hypothesis that might be tested with appropriate mutants for that system.

The mechanism of growth inhibition by uracil is unknown. The exogenous uracil might be incorporated into DNA, or it might act through its known inhibition of uracil-DNA glycosylase

(19). The latter possibility is less likely, however, because *dut ung* (glycosylase) double mutants are uracil resistant (B. Duncan, personal communication). Similarly, FUdR might act through its possibly increased incorporation into the DNA of *dut* mutants, or it might act through its inhibition of thymidylate synthetase (7), thereby decreasing the dTTP that would normally compete with dUTP for incorporation into DNA.

The apparent incompatibility between *pyrE* and *dut* mutations is currently unexplainable. The *pyrE* gene codes for orotate phosphoribosyltransferase, an essential enzyme of pyridimidine metabolism. It is not clear why we failed to construct *pyrE dut* double mutants even though we used pyrimidine-containing media.

dUTPase is required for the biosynthesis of most, if not all, of dTMP (4). About 75 to 80% of dUMP, its immediate precursor, comes from the pathway dCTP → dUTP → dUMP, and the rest comes from one or more other pathways including UDP → dUDP → dUTP → dUMP. We might expect *dut* mutants to be thymine requiring, but they were not. Unfortunately, cells that were auxotrophic at 25°C were removed from the mutagenized population (24) before our screening for the *dut* mutants. To explore the possible essential nature of the *dut* gene in dTMP biosynthesis, we have recently attempted to produce very tight mutations by extensive deletions or insertions. We could not obtain insertions of Tn*5* or Tn*10* elements into *dut*, nor could we obtain *dut* deletions promoted by nearby insertions of these elements. In our most intensive effort, we surveyed 15,000 Mu*cts* (14) lysogens on a rich medium containing additional thymidine. We found 19 hyper-Rec colonies, none of which was *dut*.

The effects of *dut* mutation are similar to those of thymine starvation. When thymineless *E. coli* are deprived of thymine, they lose viability, their DNA is broken, there is an increase in repair synthesis dependent on *polA*, and there is an increase in mutational and recombinational frequencies (28). These effects are mimicked by the exposure of prototrophs to FUdR (8), an agent to which *dut* mutants are unusually sensitive. It is possible, therefore, that thymineless death may be mediated by the misincorporation of uracil into DNA resulting from a decreased production of dTTP. A fall in dTTP levels leads to derepression of dCTP deaminase (3). Large amounts of dUTP would then be produced and incorporated into DNA without competition from dTTP.

The process by which *E. coli* makes most of its dTMP from triphosphate precursors is metabolically wasteful and entails the production of a harmful intermediate, dUTP, whose removal from DNA exacts an even higher metabolic cost. Perhaps dUTP is needed to serve an essential undiscovered function, perhaps some uracil misincorporation is desired because it promotes recombination and mutation and is thus beneficial to the cell population, or perhaps dUTP production will make more sense when we come to understand the complex regulatory controls in pyrimidine biosynthesis (5).

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