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Lethality of a *dut* (Deoxyuridine Triphosphatase) Mutation in *Escherichia coli*

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A chloramphenicol resistance gene was cloned into a plasmid-borne *dut* gene, producing an insertion mutation that was then transferred to the chromosome by allelic exchange. The mutation could not be acquired by haploid strains through substitutive recombination, even when two flanking markers were simultaneously transduced. The insertion was easily transferred, via generalized transduction, into the chromosomal *dut* region of strains harboring a λ *dut*$^+$ transducing phage; however, the resulting *dut* mutant/λ *dut*$^+$ merodiploid could not then be cured of the prophage. This apparent lethality of the mutation could not be explained by effects on adjacent genes; the *dfp* gene retained complementing activity, and a *ttk* insertion mutant was viable. The *dut* gene product, deoxyuridine triphosphatase, is known to reduce incorporation of uracil into DNA and to be required in the de novo synthesis of thymidylate. Therefore, an attempt was made to determine whether the *dut* insertion would be tolerated in strains carrying the following compensatory mutations: *dcd* (dCTP deaminase) and *cdd* (deoxycytidine deaminase), which should reduce dUTP formation; *ung* (uracil-DNA glycosylase), which should reduce fatally excessive excision repair; *deoA* (thymidine phosphorylase), which should enhance the utilization of exogenous thymidine; and *sulA*, which should reduce the lethal side effects of SOS regulon induction. These mutations, either alone or in various combinations, did not permit the survival of a haploid *dut* insertion mutant, suggesting that the *dut* gene product might have an essential function apart from its deoxyuridine triphosphatase activity.

Thymine is not an obligatory constituent of DNA; there are, for example, uracil-containing DNA bacteriophages (30). Why, then, does the DNA of almost all organisms contain thymine rather than uracil? A promising approach to this question lies in studying bacterial mutants that incorporate large amounts of uracil into their DNAs.

In *Escherichia coli* and its relative *Salmonella typhimurium*, there is considerable opportunity for the incorporation of uracil into DNA because they produce a large amount of dUTP as an obligatory intermediate in the de novo synthesis of thymidylate (23, 32). Although the DNA polymerases of *E. coli* can utilize dUTP about as well as dTTP, this is largely prevented by the enzyme deoxyuridine triphosphatase (dUTPase), which hydrolyzes dUTP to PP_i and dUMP, the substrate for thymidylate synthetase. Although *dut* (dUT Pase) mutants were expected to have uracil in their DNA, none was found. Instead, they displayed an excessive fragmentation of nascent DNA, consistent with the excision of uracil that was transiently incorporated (33). The first step in this repair pathway involves the enzyme uracil-DNA *N*-glycosylase (9), which is encoded by the *ung* gene. The glycosylase hydrolyzes the base from the DNA, leaving an apyrimidinic site that is excised by other enzymes (32). Stable incorporation of uracil into DNA is achieved only by combining mutations for the two genes. Thus, in a *dut ung* mutant, up to 20% of DNA thymine residues may be replaced by uracil (36).

The existing *dut* mutations were produced by alkylating agents (15, 16). Although the most defective allele *dut-1* produced an enzyme with less than 1% of wild-type dUT-Pase activity, the mutant cells grew well on complex media at high or low temperature. However, the residual dUTPase activity appeared to be temperature sensitive in vivo as well as in vitro; e.g., a *dut-1* mutant was a thymidine auxotroph at 42°C but not at 30°C. With a tighter mutation, we might be able to replace all DNA thymine with uracil. With this intent, we constructed an insertion mutation in *dut*.

MATERIALS AND METHODS

Nomenclature. Genetic nomenclature is that of Bachmann et al. (4), with the following exceptions. *dut-21*::*cat* (or *dut*::*cat*) is an insertion mutation that inactivates *dut* and that contains the chloramphenicol acetyltransferase gene of Tn*9*, *ttk* is our designation for a gene in the *dut* operon that codes for a 23-kilodalton (kDa) polypeptide of unknown function (17, 29), *ttk-1*::*kan* is an insertion mutation containing the kanamycin resistance (aminoglycoside 3′-phosphotransferase) gene of Tn*903*, and *dfp* is a gene affecting DNA synthesis that codes for a flavoprotein (29). Phenotypic abbreviations are derived from the gene names, except as follows: Cm, chloramphenicol; Km, kanamycin; Ap, ampicillin; Tc, tetracycline.

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. λBW111 is a thermoinducible (*c*I857) transducing phage carrying *pyrE* and *dut* genes on a 7.5-kilobase (kb) cloned fragment (31).

Media. Tryptone-yeast (TY) medium (37) was used routinely and was supplemented with 0.5 mM thymidine to enhance the growth of *dut* mutants. VB minimal media (37), NZ media (31), and nutritional supplements (37) were as described previously. The following antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; kanamycin, 40 μg/ml; tetracycline, 20 μg/ml; chloramphenicol, 10 μg/ml; and streptomycin, 200 μg/ml.

Strain construction. Transduction with bacteriophage P1 *vir* (37), bacterial conjugation (37), and transformation (19) were as described previously. Before selection for transduction of antibiotic resistance, cells were incubated in TY

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TABLE 1. *E. coli* strains and plasmids used in this study

Designation	Relevant genotype[a]	Source or reference[b]
Bacteria		
BD2008	*ung-151*::Tn*10*	B. Duncan (10)
BW283	*dut-1* Δ(*his-dcd*)*453*	This laboratory
BW284	*dut-1 dcd-2*	This laboratory
BW286	*dut-1* Δ(*xth-pncA*)*90*	32
BW287	*dut-1 xthA3*	32
BW371	*cysE pyrE*	This laboratory
BW481	*cysE*	This laboratory
ES110	*dfp-707 recA*	29
GC4540	*sulA100*::Tn*5 pyrD*	S. Gottesman (8)
HD1038	*dcd-1 leu rpsL*	J. A. Fuchs (24)
HH1	*dut-21*::*cat* (λBW111)	This study
HH3	*dut-21*::*cat* Δ(*his-dcd*)*453* (λBW111)	P1(HH1) × BW283(λBW111)
HH5	*dut-21*::*cat dcd-2* (λBW111)	P1(HH1) × BW284(λBW111)
HH6	*dut-21*::*cat* Δ(*his-dcd*)*453 ung-151*::Tn*10* (λBW111)	P1(BD2008) × HH3
HH7	*dut-21*::*cat dcd-2 ung-151*::*Tn10* (λBW111)	P1(BD2008) × HH5
HH8	*dut-21*::*cat sulA100*::Tn*5* (λBW111)	P1(GC4540) × HH1[c]
HH9	*dut-21*::*cat sulA100*::Tn*5* Δ(*his-dcd*)*453* (λBW111)	P1(GC4540) × HH3[c]
HH10	*dut-21*::*cat sulA100*::Tn*5 dcd-2* (λBW111)	P1(GC4540) × HH5[c]
HH11	*dut-21*::*cat sulA100*::Tn*5* Δ(*his-dcd*)*453 ung-151*::Tn*10* (λBW111)	P1(GC4540) × HH6[c]
HH12	*dut-21*::*cat sulA100*::Tn*5 dcd-2 ung-151*::Tn*10* (λBW111)	P1(GC4540) × HH7[c]
HH13	*ung-151*::Tn*10 cysE pyrE*	P1(BD2008) × BW371
HH14	*deoA22 dcd-1 rpsL*	Mating: SA53 × HD1038[d]
HH15	*ung-151*::Tn*10 dcd-1 rpsL*	P1(BD2008) × HD1038
HH16	*ung-151*::Tn*10 dcd-1 deoA22 rpsL*	P1(BD2008) × HH14
HH17	*deoA22 thr*::Tn*10*	P1(NK5138) × SA53
HH21	*deoA22 dcd-1 cdd-50 rpsL*	Mating: SA53 × LD175[d]
HH23	*ung-151*::Tn*10 dcd-1 cdd-50*	P1(BD2008) × LD175
HH24	*deoA22 ung-151*::Tn*10 dcd-1 cdd-50*	P1(BD2008) × HH21
LD175[e]	*dcd-1 cdd-50 leu rpsL*	J. A. Fuchs (24)
MM383	*polA12*(Ts)	20
N100	*recA*	R. N. Rao
SA53	Hfr PO-100 *deoA22*	CGSC (26)
Plasmids		
pES23	pWB1 *dfp*::Tn*1000 dut*$^+$; Ap^r	29
pES25	pWB1 *dfp*::Tn*1000 dut*$^+$; Ap^r	29
pMOB02	pBR322:Tn*9*; Cm^r Tc^r	5
pNG35	mini-pBR322::Tn*903Δ1*; Km^r Ap^r	13
pWB1	*dfp dut ttk* region cloned in a derivative of pBR322; Ap^r	31
pWB31	pWB1 *dut-21*::*cat*; Cm^r Ap^r	This study
pWB32	pWB1 *ttk-1*::*kan*; Km^r Ap^r	This study

[a] Listed are only those markers used in strain construction.
[b] P1 transductional crosses are described as follows: P1(donor) × recipient. Abbreviation: CGSC, Coli Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.
[c] To exclude transpositions, *pyrD* was cotransduced with Tn*5*, and a Km^r recombinant was then transduced to PyrD$^+$.
[d] Selection was for Leu$^+$ Str^r recombinants.
[e] Originally designated HD1038 *cdd* (24).

broth containing 20 mM citrate for 1 to 2 h to prevent reinfection during the time necessary for gene expression. *dcd* mutations were detected by resistance to 5-bromodeoxyuridine (100 μg/ml) (12) and confirmed by a spectrophotometric assay for dCTP deaminase (22). *cdd* was scored with 0.1% cytidine as the sole carbon source (14). *deoA* mutants were detected by their ability to use 5 mM thymidine as the sole carbon source (1) and confirmed by enzymatic assay (21) of sonicates of cells growing logarithmically in TY broth supplemented with 0.8 mM thymidine. Transduction of *ung*::Tn*10* involved selection for Tc^r, but because of the possible transposition of Tn*10*, *ung* mutants were verified by their ability to propagate uracil-containing λ *vir* (10) or T4 (35) phages. Lysogenization with λBW111 [*c*I857(Ts) *dut*$^+$] was performed by infection at high multiplicity (27); lysogens were confirmed by their temperature sensitivity, their resistance to λ *c*60, and their sensitivity to λ *vir*.

Heat-pulse curing. A previously described procedure (27) was modified to allow the cured segregants to grow for fewer generations and in the presence of thymidine to avoid selecting against *dut* mutant cells. Lysogens containing λ *c*I857 derivatives were grown in NZ broth at 32°C to 2 × 10^8/ml and then diluted 100-fold into fresh medium at 41°C. After 6 min, they were diluted 10-fold into TY broth containing thymidine and incubated overnight at 32°C. The curing frequency was determined by plating the cells on TY agar plus thymidine at 32 and at 42°C.

Other methods. Recombinant DNA methodology (19), enzymatic assays for dUTPase (31), and λ marker rescue experiments (27) were as described previously. Plasmids specifying Cm^r were amplified with spectinomycin (6).

RESULTS

Isolation of the *dut*::*cat* insertion. The recombinant plasmid pWB1 consists of a ColE1-derived vector containing a 3.3-kb

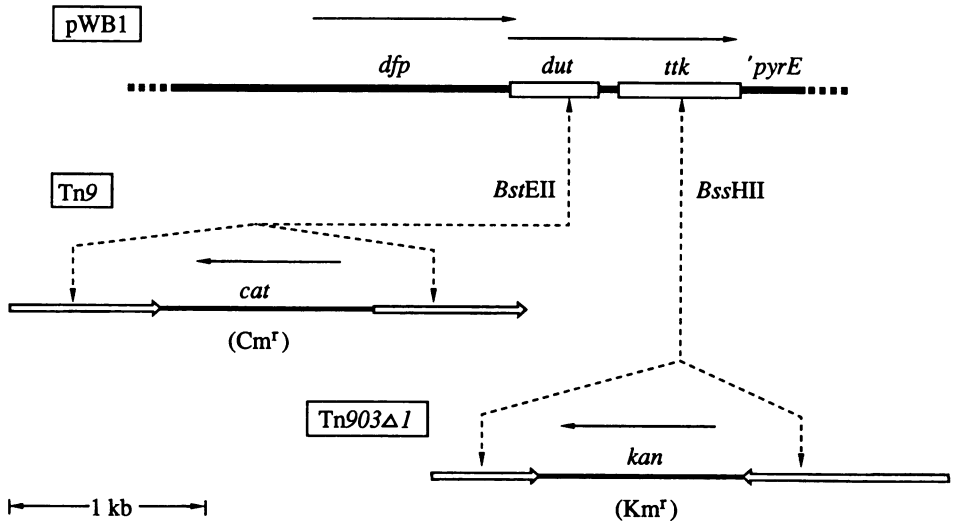

FIG. 1. Construction of *dut-21*::*cat* and *ttk-1*::*kan* insertions. At the top is the 3.3-kb segment of *E. coli* chromosomal DNA carried by plasmid pWB1. A *Bst*EII fragment of Tn*9* (from pMOB02) and a *Bss*HII fragment of Tn*903Δ1* (from pNG35) were cloned individually in the corresponding restriction sites in the *dut* and *ttk* genes of pWB1 to produce plasmids pWB31 and pWB32, respectively. Solid arrows represent transcripts, and open rectangles are the *dut* and *ttk* structural genes. *'pyrE* is the cloned 3'-terminal portion of *pyrE*. In the transposons, open arrows indicate the repeated sequences, one of which is partially deleted in Tn*903Δ1*. The transposons are oriented in the same direction as the final insertions. Map references: pWB1 and the *dfp-pyrE* region (17, 18, 29, 31); Tn*9*/IS*1* (2, 25); and Tn*903* (13).

insert from the *dut* region of the *E. coli* chromosome (Fig. 1). We found it to have only one *Bst*EII site, that predicted by the DNA sequence (18) to be at nucleotide 336 of the 449-base-pair *dut* gene. Our strategy was to clone an 1,870-base-pair *Bst*EII segment of Tn*9*, specifying Cm^r, into the *Bst*EII site within *dut*. Tn*9* consists of two similarly oriented copies of IS*1* flanking a region that contains the *cat* (chloramphenicol acetyltransferase) gene. Its *Bst*EII sites are within IS*1* (Fig. 1). Our source of the Tn*9* segment was the plasmid pMOB02, which also specifies Tc^r. The two plasmids were digested with endonuclease *Bst*EII, mixed, and treated with T4 DNA ligase. The mixture was then used to transform strain N100, selecting simultaneously for the Cm^r marker of Tn*9* and the Ap^r marker of pWB1. Three transformants that were Tc^s were picked for further study.

Endonuclease *Bst*EII produces single-stranded DNA ends that contain one unspecified nucleotide. From the known sequences of IS*1* (25) and *dut* (18), it was evident that only one orientation of the insert would permit full complementarity between the joined ends. When the recombinant plasmids were analyzed by digestion with restriction endonucleases *Bst*EII, *Pvu*II, and *Hin*fI, the data (not shown) were consistent with insertions into *dut* and only in the predicted orientation, that shown in Fig. 1.

The insertion mutation, designated *dut-21*::*cat*, was then transferred from a plasmid to the chromosome. The *polA*(Ts) strain MM393 was transformed with plasmid pWB31 (*dut-21*::*cat*) and grown in the presence of chloramphenicol at alternating high and low temperatures to enrich for the desired recombinants (7). P1 phages were then grown on the culture and used to transduce the mutation to other strains. In preliminary experiments, it was found that even at 32°C the desired recombinant could be obtained only if the recipient had more than one copy of the dut^+ gene. Therefore, we used a recipient that contained a λ dut^+ transducing prophage, λBW111. To facilitate subsequent curing, the prophage also contained a *c*I857(Ts) mutation. Accordingly, the *dut-21*::*cat* insertion was transferred via P1-mediated generalized transduction to the *cysE* mutant BW481 (λBW111). Cys^+ Cm^r recombinants were selected at 32°C. Because *cysE* was not on the prophage, we could be reasonably certain that the insertion was transferred to the chromosomal copy of *dut* by cotransduction with the nearby *cysE* gene. Of 200 transductants screened, 2 were Ap^s, i.e., plasmid-free. One such strain, HH1, was chosen for further study.

Inability to cure a *dut*::*cat*/λ *dut*$^+$ strain. Strain HH1 was a *dut*::*cat*/λ dut^+ merodiploid. It had an insertion mutation at the normal chromosomal location of *dut*, i.e., near 82 min on the linkage map (4). It also possessed a functional copy of the gene on an integrated λ prophage at 17 min. If dUTPase is not needed for viability, then we should be able to cure the merodiploid and produce a haploid *dut*::*cat* mutant. Because the phage bore a *c*I857(Ts) mutation, it was susceptible to heat-pulse curing, a procedure in which transient derepression of the prophage leads to its excision without significant replication. The extrachromosomal, nonreplicating prophage should then become diluted in the cell population during further growth. To estimate the efficiency of curing of the thermoinducible prophage, we simply determined the fraction of cells that were no longer temperature sensitive after this treatment. Because dUTPase-deficient cells are thymidine auxotrophs (32), 0.5 mM thymidine was added to all growth media. The apparent curing efficiency for a lysogen of strain BW481 (dut^+/λ dut^+) was 35%, whereas that for strain HH1 (*dut*::*cat*/λ dut^+) was 0.01 to 0.1%. We considered the possibility that *dut-21*::*cat* might specify a temperature-sensitive phenotype, in which case temperature resistance may not be used as the sole criterion for the curing of HH1. Therefore, after heat-pulse treatment of HH1, 48 temperature-sensitive clones were tested, and all were confirmed to be λ lysogens at 32°C by virtue of their resistance to λ *c*60 and their sensitivity to λ *vir*.

The apparently cured (temperature resistant) derivatives of HH1, which arose at a frequency of $<10^{-3}$, were analyzed further. Of 12 colonies studied, 4 were still Cm^r and were dUTPase proficient by enzymatic assay, but they were found by a marker rescue experiment to have retained the λ *L*

TABLE 2. Inability to cotransduce *dut*::*cat* with *cysE*$^+$ when the recipient has only one *dut* gene[a]

cys$^+$ recombinants	No. of crossovers[b]	Transductants (%) of:	
		BW371(pWB1) (*cysE dut*$^+$ *pyrE*/*dut*$^+$)	BW371 (*cysE dut*$^+$ *pyrE*)
Cm^r Pyr$^+$	2	12.5	0
Cm^r Pyr$^-$	2	0.8	0
Cm^s Pyr$^+$	4	0.3	0.5
Cm^s Pyr$^-$	2	86.6	99.5

[a] P1-mediated generalized transduction was performed at 37°C with strain HH1 (*dut*::*cat*/λ *dut*$^+$) as the donor. Cys$^+$ transductants (252 and 200, respectively) were selected in each cross and scored for Cm^r (*dut*::*cat*) and for uracil dependence (*pyrE*).

[b] Based on the known sequence *cysE-dut-pyrE* (4).

gene, suggesting that they possessed cryptic λ *dut*$^+$ prophages. Their temperature resistance resulted, therefore, from a prophage mutation. The eight other colonies were Cm^s, suggesting that before curing they underwent a recombination between prophage and chromosomal *dut* genes on sister chromosomes to produce a *dut*$^+$/λ *dut*$^+$ merodiploid. This finding provided an internal control for the experiment, demonstrating that it was specifically the makeup of the *dut* genes of strain HH1 that affected its curing. Our overall failure to obtain haploid *dut*::*cat* derivatives by prophage curing implies that such mutants are inviable.

Transduction of *dut*::*cat*. The insertion mutation was isolated in merodiploid strains that had at least one functional copy of the *dut* gene. The mutation could be easily transferred via phage P1-mediated generalized transduction to another *dut*$^+$/*dut*$^+$ merodiploid, BW371(pWB1) (Table 2). The recipient had single, mutant copies of *pyrE* and *cysE*. Of the *cysE*$^+$ transductants of BW371(pWB1), 13.3% had coinherited *dut*::*cat* and 12.8% had coinherited *pyrE*$^+$. The results are consistent with the current linkage map (4) in which the gene order is *cysE dut pyrE* and in which the closely linked *dut* and *pyrE* genes are about 0.8 min from *cysE*. A three-point analysis of the results is also consistent with the known gene order if one assumes that the rarest class of recombinants, *cysE*$^+$ *dut*$^+$ (Cm^s) *pyrE*$^+$, arose from four crossovers instead of two, i.e., from the inheritance of the two outer markers from the donor parent. However, different results were obtained when the recipient was the haploid, plasmid-free BW371 (Table 2). First, no *dut*::*cat* transductants were found. Second, only one (0.5%) *pyrE*$^+$ recombinant was seen, despite the normally high linkage between *pyrE* and *dut*. Third, the four-crossover class was larger than some of the two-crossover classes; although *dut*::*cat* was not inherited, the flanking markers were. The lethality of the *dut* insertion mutation was thus confirmed by our inability to transfer it via substitutive recombination into a haploid *dut*$^+$ cell.

***ttk* mutations.** The *dut* gene is known to be the first member of a two-gene operon. The second, which we hereby designate *ttk*, codes for a 23-kDa protein of unknown function (17, 29). Evidence for the existence of the operon is based on DNA sequence analysis (18) and on the polar effects of insertion mutations. For example, *dut*::Tn*1000* mutations in plasmid pWB1 eliminated observable production of the 23-kDa polypeptide in maxicell preparations (29). Similarly, the *dut*::*cat* insertion might also affect *ttk*. If *ttk* were an essential gene, then the lethality of *dut*::*cat* might be the result of its effect on *ttk* expression rather than on dUTPase production. To test this hypothesis, we isolated a *ttk* insertion mutation.

We cloned a 1.6-kb *Bss*HII segment of Tn*903*, which specifies kanamycin resistance, into the *ttk* gene on plasmid pWB1 (Fig. 1). The plasmid contained a single *Bss*HII site at nucleotide 258 in the 633-base-pair open reading frame of *ttk* (18). The Tn*903* segment was purified by gel electrophoresis from a digest of plasmid pNG35, mixed with a phosphatase-treated *Bss*HII digest of pWB1, and incubated with T4 DNA ligase. The mixture was then used to transform strain N100 to Ap^r and Km^r. Plasmids were isolated that bore insertions in both orientations, as confirmed by digestion with endonuclease *Hin*dIII.

We tested one of these plasmids, pWB32 (*dut*$^+$ *ttk-1*::*kan*), for its ability to complement a *dut*::*cat* mutant. After strain HH1 (*dut*::*cat*/λ *dut*$^+$) was transformed by the plasmid, it could be cured of the λ prophage with 32% efficiency. Therefore, a functional *dut* gene was necessary and sufficient to restore viability to the *dut* insertion mutant. The *ttk-1*::*kan* mutation was then transferred to the chromosome by the same method used for the *dut* insertion. Subsequently, the mutation was easily cotransduced with *cysE*$^+$ into strain BW481, and from there into BW371. The recombinants were stable. They grew well on both minimal and rich media and had no discernible phenotype apart from Km^r. Because a *ttk* insertion mutant is viable, it is unlikely that the lethality of the *dut* insertion can be attributed to its effect on the expression of *ttk*.

***dut*::*cat* does not affect *dfp*.** The *dfp* gene (Fig. 1) coincides with an open reading frame that overlaps *dut* by 17 nucleotides (18, 28, 29). Its transcription is codirectional with but independent of that of *dut* (29). It is an essential gene that codes for a flavoprotein of unknown function, and mutations in the gene affect the synthesis of DNA and of pantothenate. It was possible that a new transcript originating from the *dut* insertion might extend into *dfp* and block its expression. We used complementation analysis to test the functionality of the neighboring *dfp* gene. Strain ES110 [*dfp-707*(Ts) *recA*] was independently transformed with three insertion mutants of plasmid pWB1: pWB31 (*dut*::*cat*), pES23 (*dfp*::Tn*1000*), and pES25, (*dfp*::Tn*1000*). Nine transformants were studied, three for each plasmid. The *dut*::*cat* plasmid fully complemented the *dfp*(Ts) mutant (>90% survival at 42°C), whereas the controls bearing *dfp* insertions failed to do so (<1% survival). Therefore, the lethality of *dut*::*cat* cannot be explained by an effect on the *dfp* gene.

***dut*::*cat* combined with other mutations.** A tight *dut* mutation would be expected to produce a high level of uracil substitution for thymine in DNA. Previous work has suggested two major possibilities for the inviability of cells containing such DNA. First, extensive excision repair of uracil-containing DNA is lethal. It has been observed, for example, that DNA phages that acquire a high uracil content (via growth on *dut ung* hosts) did not survive in wild-type cells, but grew in repair-deficient (*ung*) mutants (9). Second, the substitution of uracil for thymine might inactivate some essential protein recognition sequences in DNA. This effect has already been observed in a nonvital region, the *lac* operator (9). For these reasons, a *dut*::*cat* allele might be nonlethal in a mutant having reduced synthesis of dUTP (and, hence, of uracil-containing DNA) or in one that has a block in the excision repair of uracil-containing DNA.

Our choice of such mutants was based on the metabolic scheme shown in Fig. 2. *dcd* mutations were studied because dCTP deaminase is believed to produce 70 to 75% of cellular dUTP (22). A mutation in *cdd*, the gene for the salvage

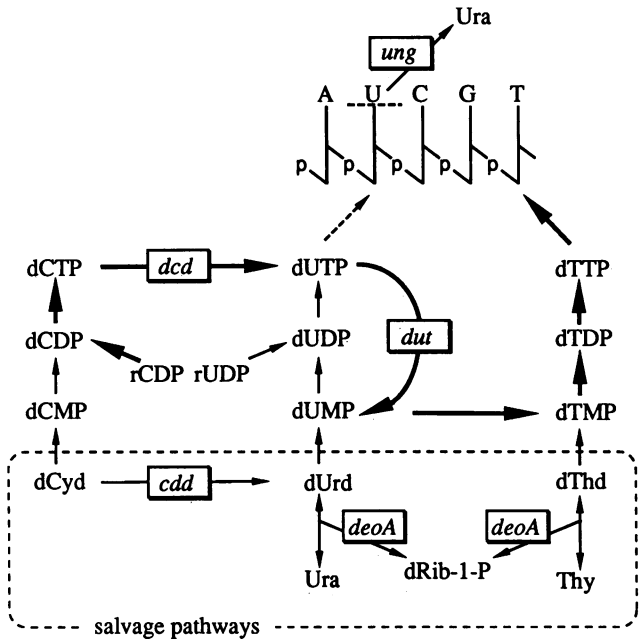

FIG. 2. Metabolism of dUTP in *E. coli*. Arrows indicate directions of flow within the overall pathway rather than the equilibrium of individual reactions. Bold arrows indicate the major pathway. Boxes contain the structural genes of interest: *ung*, uracil-DNA *N*-glycosylase; *dut*, dUTPase; *dcd*, dCTP deaminase; *cdd*, deoxycytidine (cytidine) deaminase; and *deoA*, thymidine (deoxyuridine) phosphorylase. Abbreviations: dCyd, deoxycytidine; dUrd, deoxyuridine; dThd, deoxythymidine; dRib-1-P, deoxyribose-1-phosphate.

enzyme deoxycytidine deaminase, should further reduce dUTP formation. An *ung* (uracil-DNA glycosylase) mutation was used because it blocks the first step in uracil excision repair of DNA and thus permits the stable incorporation of uracil into DNA (9, 36). We also considered the possibility that the breakage of DNA during uracil excision repair (33) might trigger the SOS response, leading to an inhibition of cell division mediated by the *sfi* genes (34). Accordingly, we tried a *sulA* (*sfiA*) mutation, which is known to enhance the survival of SOS-induced cells. Because *dut* mutants are thymidine auxotrophs (32), all media were supplemented with 0.5 mM thymidine, and in some experiments, a *deoA* (thymidine phosphorylase) mutation was provided to enable its more efficient utilization. In each case, we tested either the curing of a *dut*::*cat*/λ *dut*$^+$ derivative or the ability to transduce the appropriate mutant to Cm^r using a *dut*::*cat* donor.

None of the mutations tested, either alone or in combination, provided a genetic background that would tolerate a homoallelic *dut*::*cat* mutation (Table 3). Under conditions in which we obtained >30% curing of a *dut*$^+$/λ *dut*$^+$ lysogen, the apparent curing frequency for its *dut*::*cat*/λ *dut*$^+$ counterpart was less than 0.5%. In P1 transduction experiments, we observed no Cm^r recombinants of haploid *dut*$^+$ strains under conditions in which a merodiploid (*dut*$^+$/λ *dut*$^+$) produced 50 to 200 transductants per plate after 1 day of incubation. Because a tight dUTPase-deficient mutant might grow poorly, the plates were incubated for at least 2 more days, during which some of the haploid recipient strains did yield a few minute Cm^r colonies. However, these recombinants had normal levels of dUTPase and were genetically unstable; after a single passage in the absence of chloramphenicol, >20% of the cells in each culture became Cm^s. These properties of the rare dUTPase-proficient Cm^r transductants were consistent with those of strains containing both *dut*$^+$ and *dut*::*cat* alleles within a tandem duplication of the *dut* region (3).

TABLE 3. Strains failing to yield haploid *dut*::*cat* derivatives

Strain	Relevant mutations[a]	Method attempted[b]
HH1		Curing
BW371		Transduction
HH3	Δ*dcd*	Curing
BW283	Δ*dcd*	Transduction
HH5	*dcd-2*	Curing
BW284	*dcd-2*	Transduction
HD1038	*dcd-1*	Transduction
LD175	*dcd-1 cdd*	Transduction
HH13	*ung*	Transduction
HH6	*ung* Δ*dcd*	Curing
HH7	*ung dcd-2*	Curing
HH15	*ung dcd-1*	Transduction
HH23	*ung dcd-1 cdd*	Transduction
HH17	*deoA*	Transduction
HH14	*deoA dcd-1*	Transduction
HH21	*deoA dcd-1 cdd*	Transduction
HH16	*deoA dcd-1 ung*	Transduction
HH24	*deoA dcd-1 cdd ung*	Transduction
HH8	*sulA*	Curing
HH9	*sulA* Δ*dcd*	Curing
HH10	*sulA dcd-2*	Curing
HH11	*sulA* Δ*dcd ung*	Curing
HH12	*sulA dcd-2 ung*	Curing

[a] *dut-21*::*cat* and λBW111(*dut*$^+$) were additionally present in those strains that were tested by curing.

[b] λ*dut*$^+$/*dut*::*cat* merodiploids were tested for their ability to be cured of the λ *dut*$^+$ prophage. Haploid *dut*$^+$ strains were tested for their ability to acquire the *dut*::*cat* mutation by generalized transduction at 37°C. Criteria are discussed in the text.

Exogenous thymidine and *deoA*. In our experiments, all media were supplemented with 0.5 mM thymidine, including the complex TY medium. It was hoped that the thymidine would be utilized by thymidylate kinase to yield large amounts of dTMP and, subsequently, dTTP. This consequence should enable the survival of tight *dut* mutants for two reasons. First, it should compensate for their block in the de novo synthesis of dTMP, which is derived from dUTP (Fig. 2); and second, the resulting dTTP might competitively inhibit the incorporation of dUTP into DNA. In view of our failure to obtain haploid *dut* insertion mutants, we decided to test these premises.

xth (exonuclease III) mutants are deficient in the excision repair of uracil-containing DNA; therefore, *dut-1 xth* strains are temperature-sensitive, conditional lethal mutants (32). The lethality can be reversed by the addition of a *dcd* mutation (32), which reduces the formation of dUTP and, hence, its subsequent incorporation into DNA (Fig. 2). If exogenous thymidine also blocks uracil misincorporation, it, too, should restore the viability of a *dut-1 xth* mutant. We tested strains BW286 (*dut-1 xth-3*) and BW287 (*dut-1* Δ*xth*) and found that exogenous thymidine, at levels of up to 0.5 mM, was ineffective; survival of these strains on TY medium at 42°C was still less than 1%. However, the thymidine might be poorly utilized because of cleavage by the periplasmic enzyme thymidine phosphorylase, which releases deoxyribose-1-phosphate that is then rapidly metabolized (23). To increase thymidine utilization we introduced *deoA* (thymidine phosphorylase) mutations into the *dut-1 xth* mutants.

deoA22 was transferred from strain HH17 via cotransduction with a nearby Tn*10* at 32°C. We picked eight Tc^r transductants of strain BW286 [*dut-1* Δ(*xth-pncA*)*90*] and nine of BW287 (*dut-1 xthA3*). Two of the former and five of the latter were temperature resistant; their survival at 42°C on TY agar medium was 40 to 100%. Under the same conditions, the parental *dut xth* strains had a survival of <1%. Among the transductants, there was a one-to-one correspondence between temperature resistance and thymidine phosphorylase deficiency, and the temperature resistance was apparent even without additional thymidine in the TY medium. Therefore, a *deoA* mutation could reverse the conditional lethality of *dut-1 xth* mutants, whereas a high concentration of thymidine could not.

We then asked whether a *deoA* mutation could enable the survival of a *dut* insertion mutant in media supplemented with thymidine. We found that it did not do so, either alone or in combination with *ung*, *dcd*, and *cdd* mutations (Table 3).

DISCUSSION

To produce a tight mutation in *dut*, we chose to insert an antibiotic resistance cassette. The antibiotic resistance marker not only permitted us to select transductants easily but also to detect the mutant allele in the presence of a functional one, which we could not do reliably with the dUTPase assay alone. We found that the *dut*::*cat* mutation could not exist in a homoallelic state; it could be transduced only into a *dut*$^+$/*dut*$^+$ merodiploid, such as a wild-type strain harboring a λ *dut*$^+$ prophage or a *dut*$^+$ plasmid. Moreover, a *dut*::*cat*/λ *dut*$^+$ strain could not be cured of the prophage to yield a haploid, dUTPase-deficient, *dut*::*cat* derivative. When we attempted to transduce the *dut*::*cat* mutation into a haploid *dut*$^+$ strain, we succeeded only in transferring one or both of two flanking markers without cotransducing Cm^r. From these data, the *dut*::*cat* insertion appears to be both recessive and lethal. We then found that the lethality of *dut*::*cat* could not be attributed to its effects on its neighboring genes. *dfp* gene function was unaffected in complementation assays, and an insertion mutation in *ttk* was nonlethal. Therefore, the lethality must be attributed directly to the *dut* mutation.

Strains bearing *dut-1*, a revertable point mutation, have less than 1% of wild-type levels of dUTPase at 37°C (32), and yet they are viable; however, a presumed null mutant, one bearing *dut*::*cat*, is not viable. Although mutant enzymes may appear to be much less active in vitro than they are in vivo, several observations suggest that this is not true for the *dut-1* enzyme. *dut-1* mutants have an absolute requirement for thymidine at 42°C, they have an enhanced frequency of intrachromosomal recombination, and they display delayed septation (i.e., minor filamentation) during growth (32). Therefore, only a small fraction of wild-type dUTPase activity may be required for viability.

The known properties of strains having point mutations like *dut-1* suggested two likely hypotheses to explain the lethality of an insertion mutation. The first is that extensive excision repair of the resulting uracil-containing DNA should produce lethal double-strand breaks. This possibility is supported by the observations that (i) viable *dut* mutants display excessive fragmentation of nascent DNA (33), (ii) phages that contain uracil in their DNA (e.g., λ grown on a *dut ung* mutant) are unable to replicate in wild-type cells but can be propagated on the repair-deficient *ung* hosts (9), and (iii) the stable transfer of uracil-containing DNA by conjugation between a *dut ung* donor and an *ung*$^+$ recipient is similarly restricted (36). The second major hypothesis is that uracil residues may interfere with the binding to DNA of specific proteins needed for its replication or for the control of essential genes. It is suggested by the observation that limited uracil incorporation interferes with the *lac* operon, whose operator contains a thymine that is needed to bind the repressor (11). Thus, *dut-1 ung* mutants, which have uracil stably incorporated into their DNAs, appear to be Lac constitutive because a significant fraction of the cells in a colony would be expected to have uracil substituted in that position (9). However, if thymines are indeed needed at critical protein recognition sites in some essential genes, there cannot be many such genes because *dut-1 ung* mutants are viable even when as much as 20% of their DNA thymine residues are replaced by uracil (9, 36). Because uracil is frequently misincorporated in place of thymine in the DNA of wild-type cells (36), there may be a selective pressure against the evolution of such thymine-requiring sites in vital genes.

In consideration of the possibilities presented above, we tested a series of mutants defective in dUTP synthesis or in DNA-uracil repair, but we found that they still could not host the *dut*::*cat* mutation. Two of these mutations, *dcd* and *ung*, were known to reverse the conditional lethality of *dut-1 xth* mutants, which are deficient in the repair of uracil-containing DNA (32). We found a third mutation, affecting thymidine phosphorylase (*deoA*), that also produced phenotypic reversion of a *dut xth* strain, presumably by permitting the more efficient conversion of exogenous thymidine to dTTP, which would compete with dUTP for incorporation into DNA. None of these mutations permitted the survival of the *dut*::*cat* mutant. Our results strengthen the likelihood of alternative explanations whereby the inviability of a *dut* insertion mutant may be unrelated to uracil in DNA. Thus, dUTPase may be needed for the stability of an essential enzyme complex of which it is a part; or dUTPase may have an alternative function, e.g., to transfer dUMP residues to acceptors other than water via an enzyme-dUMP intermediate. The latter hypothesis could explain why *E. coli* goes out of its way to make dTMP via dCTP and dUTP, a process that wastes a great deal of energy for no apparent reason.

Our findings suggest that it should be possible to produce temperature-sensitive lethal mutations in *dut*. By studying the cells after a temperature shift, and by isolating and identifying second-site phenotypic revertants, we should then gain valuable insights into the function of dUTPase.

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LITERATURE CITED

1. **Ahmad, S. I., and R. H. Pritchard.** 1969. A map of four genes specifying enzymes involved in catabolism of nucleosides and deoxynucleosides in *Escherichia coli*. Mol. Gen. Genet. **104**:351–359.
2. **Alton, N. K., and D. Vapnek.** 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature (London) **282**:864–869.
3. **Anderson, R. P., and J. R. Roth.** 1977. Tandem genetic duplications in phage and bacteria. Annu. Rev. Microbiol. **31**:473–505.
4. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.

5. **Bittner, M., and D. Vapnek.** 1981. Versatile cloning vectors derived from the runaway-replication plasmid pKN402. Gene **15**:319–329.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
7. **Cunningham, R. P., and B. Weiss.** 1985. Endonuclease III (*nth*) mutants of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82**:474–478.
8. **D'Ari, R., and O. Huisman.** 1983. Novel mechanism of cell division inhibition associated with SOS response in *Escherichia coli*. J. Bacteriol. **156**:243–250.
9. **Duncan, B. K.** 1981. DNA glycosylases, p. 565–586. *In* P. D. Boyer (ed.), The enzymes, vol. 1. Academic Press, Inc., New York.
10. **Duncan, B. K.** 1985. Isolation of insertion, deletion, and nonsense mutations of the uracil-DNA glycosylase (*ung*) gene of *Escherichia coli* K-12. J. Bacteriol. **164**:689–695.
11. **Fisher, E. F., and M. H. Caruthers.** 1979. Studies on gene control regions. XII. The functional significance of a Lac operator constitutive mutation. Nucleic Acids Res. **7**:401–416.
12. **Fuchs, J. A., and O. Karlström.** 1976. Mapping of *nrdA* and *nrdB* in *Escherichia coli* K-12. J. Bacteriol. **128**:810–814.
13. **Grindley, N. D. F., and C. M. Joyce.** 1980. Genetic and DNA sequence analysis of the kanamycin resistance transposon Tn*903*. Proc. Natl. Acad. Sci. USA **77**:7176–7180.
14. **Hammer-Jespersen, K., and A. Munch-Petersen.** 1973. Mutants of *Escherichia coli* unable to metabolize cytidine: isolation and characterization. Mol. Gen. Genet. **126**:177–186.
15. **Hochhauser, S. J., and B. Weiss.** 1978. *Escherichia coli* mutants deficient in deoxyuridine triphosphatase. J. Bacteriol. **134**:157–166.
16. **Konrad, E. B., and I. R. Lehman.** 1975. Novel mutants of *Escherichia coli* that accumulate very small DNA replicative intermediates. Proc. Natl. Acad. Sci. USA **72**:2150–2154.
17. **Lundberg, L. G., O. H. Karlström, and P. O. Nyman.** 1983. Isolation and characterization of the *dut* gene of *Escherichia coli*. II. Restriction enzyme mapping and analysis of polypeptide products. Gene **22**:127–131.
18. **Lundberg, L. G., H.-O. Thoresson, O. H. Karlström, and P. O. Nyman.** 1983. Nucleotide sequence of the structural gene for dUTPase of *Escherichia coli* K-12. EMBO J. **2**:967–971.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Monk, M., and J. Kinross.** 1972. Conditional lethality of *recA* and *recB* derivatives of a strain of *Escherichia coli* K-12 with a temperature-sensitive deoxyribonucleic acid polymerase I. J. Bacteriol. **109**:971–978.
21. **Munch-Petersen, A.** 1975. On the catabolism of deoxyribonucleosides in cells and cell extracts of *Escherichia coli*. Eur. J. Biochem. **6**:432–442.
22. **Neuhard, J., and E. Thomassen.** 1971. Deoxycytidine triphosphate deaminase: identification and function in *Salmonella typhimurium*. J. Bacteriol. **105**:657–665.
23. **O'Donovan, G. A.** 1977. Thymidine metabolism in bacteria., p. 219–253. *In* I. Molineux and M. Kohiyama (ed.), DNA synthesis, present and future. Plenum Publishing Corp., New York.
24. **O'Donovan, G. A., G. Edlin, J. A. Fuchs, J. Neuhard, and E. Thomassen.** 1971. Deoxycytidine triphosphate deaminase: characterization of an *Escherichia coli* mutant deficient in the enzyme. J. Bacteriol. **105**:666–672.
25. **Ohtsubo, H., and E. Ohtsubo.** 1978. Nucleotide sequence of an insertion element, IS1. Proc. Natl. Acad. Sci. USA **75**:615–619.
26. **Pritchard, R. H., and S. J. Ahmad.** 1971. Fluorouracil and the isolation of mutants lacking uridine phosphorylase in *Escherichia coli*: location of the gene. Mol. Gen. Genet. **111**:84–88.
27. **Shimada, K., R. A. Weisberg, and M. E. Gottesman.** 1972. Prophage lambda at unusual chromosomal locations. I. Location of the secondary attachment sites and the properties of the lysogens. J. Mol. Biol. **63**:483–503.
28. **Spitzer, E. D., H. E. Jimenez-Billini, and B. Weiss.** 1988. β-Alanine auxotrophy associated with *dfp*, a locus affecting DNA synthesis in *Escherichia coli*. J. Bacteriol. **170**:872–876.
29. **Spitzer, E. D., and B. Weiss.** 1985. *dfp* gene of *Escherichia coli* K-12, a locus affecting DNA synthesis, codes for a flavoprotein. J. Bacteriol. **164**:994–1003.
30. **Takahashi, I., and J. Marmur.** 1963. Replacement of thymidylic acid by deoxyuridylic acid in the deoxyribonucleic acid of a transducing phage for *Bacillus subtilis*. Nature (London) **107**:794–795.
31. **Taylor, A. F., P. G. Siliciano, and B. Weiss.** 1980. Cloning of the *dut* (deoxyuridine triphosphatase) gene of *Escherichia coli*. Gene **9**:321–336.
32. **Taylor, A. F., and B. Weiss.** 1982. The role of exonuclease III in the base-excision repair of uracil-containing DNA. J. Bacteriol. **151**:351–357.
33. **Tye, B.-K., P. O. Nyman, I. R. Lehman, S. Hochhauser, and B. Weiss.** 1977. Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA. Proc. Natl. Acad. Sci. USA **74**:154–157.
34. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. **48**:60–93.
35. **Warner, H. R., and B. K. Duncan.** 1978. *In vivo* synthesis and properties of uracil-containing DNA. Nature (London) **272**: 32–34.
36. **Warner, H. R., B. K. Duncan, C. Garrett, and J. Neuhard.** 1981. Synthesis and metabolism of uracil-containing deoxyribonucleic acid in *Escherichia coli*. J. Bacteriol. **145**:687–695.
37. **White, B. J., S. J. Hochhauser, N. M. Cintrón, and B. Weiss.** 1976. Genetic mapping of *xthA*, the structural gene for exonuclease III in *Escherichia coli* K-12. J. Bacteriol. **126**:1082–1088.