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Conditional Mutator Gene in *Escherichia coli*: Isolation, Mapping, and Effector Studies

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A mutator gene, *mutD5*, whose phenotype is conditional, has been identified in *Escherichia coli*. By P1 transduction it has been shown to lie at about 5.7 min on the chromosome, being co-transduced with *proA* and *argF*. In rich medium, streptomycin- and nalidixic acid-resistant mutation frequencies are 50 to 100 times higher than those in minimal medium. In minimal medium, the *mutD5*-induced mutation frequencies are still 50 to 100 times above co-isogenic wild-type (*mut*$^+$) levels. Similar results were obtained with all markers tested. Mutant frequencies can be raised by thymidine in the medium at concentrations as low as 0.04 μM, or by the endogenous generation of thymidine from thymine plus a deoxyribosyl donor. Deoxyadenosine, various ribonucleosides, thymine, and 2-deoxyribose do not stimulate mutation. None of these effects are related to growth rate, since growth rate and mutation rate can be decoupled completely.

Spontaneous mutation rates in various organisms can deviate from the species norm owing to the presence of mutator and antimutator genes. On the one hand, organisms carrying mutator genes offer an opportunity to study the selective advantages conferred by different mutation rates in fluctuating and stable environments (Cox and Gibson, in preparation). On the other hand, mutator genes provide a probe for studying biologically important steps in gene replication in the living cell, since unusually high levels of spontaneous mutation provide examples of abnormal deoxyribonucleic acid (DNA) replication. None of the six *Escherichia coli* mutator genes now known (17, 23, 32, 33, 37, 42) has been identified with any of the known DNA synthesis genes, and none has yet been explained enzymologically.

Some *E. coli* mutator genes cause specific changes in DNA sequence. For instance, *mutS3* (formerly designated *mutS1*) produces bi-directional transitions and appears to be sensitive to base sequence, since it mutates one A:T base pair but not another located less than 50 nucleotides away (7). As another example, *mutT1* causes a single error—the unidirectional transversion A:T → C:G—and acts on coliphage lambda only during DNA replication (6, 8, 40). Specificities such as these indicate that *E. coli* employs currently undetected systems to achieve the accurate reproduction of its genetic information.

We have identified a new mutator gene in *E. coli*, designated *mutD*. It requires a small effector molecule, either thymidine or a thymidine derivative, for full mutator activity. Its identification, mapping, and characterization are the subjects of this report.

MATERIALS AND METHODS

All solutions were made in distilled, deionized water unless otherwise stated. Percentages are weight per volume.

Media. Saline used for dilution and washing was 0.85% NaCl. LB medium contained 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. T broth contained 1% tryptone (Difco) and 0.5% NaCl. VB medium is the minimal medium E of Vogel and Bonner (38). VB medium always contained 1 μg of thiamine-hydrochloride per ml. For carbon and energy source, glucose or glycerol was used at the concentrations indicated in the text. NM10 medium is VB minimal medium containing 0.2% glucose, 1 μg of thiamine-hydrochloride per ml, 50 μg each of 19 L-amino acids per ml (no asparagine; DL-serine added at 100 μg/ml), and 50 μg each of adenine, cytosine, uracil, and thymine per ml. For plates, the above media were solidified with 2% agar (Difco). VB plates always contained 0.2% glucose. For the preparation and titering of P1 lysates, LB plates contained 0.1% glucose, 1% agar, and 2.5 mM $CaCl_2$ (added just before pouring). Top layer agar consists of distilled water solidified with 0.85% agar (Difco), and was used for the measurement of mutation frequency. LB medium solidified with 0.85% agar and containing 2.5

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mM $CaCl_2$ was used for P1 lysates. Required amino acids were supplied at 20 μg/ml to liquid and solid media. NAL plates consist of T broth solidified with 2% agar and containing 50 μg of nalidixic acid (NAL) per ml, added in a few milliliters of 0.2 N NaOH; dihydrostreptomycin sulfate (DSM) plates are similar, but contain 150 μg of DSM/ml.

Chemicals. NAL was the gift of S. Archer, Sterling Winthrop Research Institute, Rensselaer, N.Y. Other sources were: DSM (Sigma), rifampin (RIF) (Ciba); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NNG) (Aldrich); deoxyadenosine (dAdo), putrescine, spermine, and spermidine (Calbiochem); amino acids, purines, pyrimidines, adenosine, cytidine, guanosine, uridine, deoxycytidine (dCyd), deoxyguanosine (dGuo), thymidine (dThd), deoxyuridine (dUrd), 2-deoxyribose, ribose, 3',5'-cyclic adenosine monophosphate, and thiamine-hydrochloride (Sigma).

Transducing and detecting the mutD5 allele. Plvir was used essentially as described by Lennox (22). Prior to adsorption, phage were ultraviolet (UV) irradiated at 57 cm from a 15-W General Electric germicidal lamp. (Other details are described in reference 7.) Adsorption was carried out at several multiplicities, and the lowest multiplicity yielding sufficient recombinants was used. Infected cells were thoroughly washed with 0.1 M sodium citrate, pH 7.0, before plating. To obtain mapping data, all transductants between a randomly drawn straight line and the edge of the plate were picked and purified by restreaking on the same selective medium. A single isolated colony from each streak was then used for further analysis. Mut$^-$ clones were detected by applying approximately 0.05 ml of an overnight LB culture on DSM and on NAL agar. The plates were then incubated about 48 h at 37 C. Strains carrying *mutD5* gave thirty to several hundred colonies per application on DSM, and fifty to many hundred on NAL; Mut$^+$ strains gave none to a few on each. Occasional recombinants that gave conflicting results on both kinds of agar were repurified and retested before being scored. None persisted in ambiguous behavior. When a Str^R strain was used as a recipient. NAL plates alone were employed.

Hfr and F' crosses. Cells were mated as described by Low (24).

Mutation frequencies. Total cell numbers were determined on LB plates, and the mutant titer on drug plates or VB plates with the appropriate amino acid omitted. Plating was done either by spreading 0.1 ml or by overlaying 0.1 to 1.0 ml in 2.5 ml of the top layer agar. VB plates were incubated for 50 h at 37 C then refrigerated at 4 C until counted. For auxotrophic reversion frequencies, 0.5×10^9 to 10^9 cells were filtered on prewashed membrane filters (Millipore Corp.), washed with two 10-ml portions of saline, resuspended in 1.5 to 2.5 ml saline, and plated at appropriate dilutions.

Growth conditions. Cells were grown with shaking in shallow layers in flasks at 37 C. Turbidity measurements to determine the mass doubling time were done with side-arm culture flasks and a Klett Summerson colorimeter, using a red filter. Cells were also grown in culture tubes which were aerated by rapid gyration or shaking at 37 C.

RESULTS

Identification and mapping of mutD5. An exponentially growing culture of strain KD1006 was mutagenized in LB medium with 1.5 mg of NNG/ml. Cys$^+$ survivors were selected in an attempt to enrich for new alleles of the *cysC*-linked *mutS* gene (7), since NNG frequently causes multiple, closely linked mutations (15). Five mutator strains were detected by replicating large patches of cells onto DSM and NAL agar. These strains gave resistant colonies under conditions where wild-type controls gave none. One, carrying an allele designated *mut-5*, was singled out for further study because the original isolate, strain KD1024, mutated rapidly only at low temperature and in rich medium. The temperature dependence was soon separated by genetic recombination, but in a series of four successive P1 transductions with *proA* or *argF* (cf. pedigree of strain KD1087 in Table 1), the Mut$^-$ phenotype was never observed to segregate from the medium-dependent phenotype. Nor has it been observed to segregate in about 10 similar crosses. In each cross, several of the Mut$^-$ progeny were shown to have low mutator activity when grown in VB medium and high mutator activity when grown in LB medium. This conditionality will be covered in detail below.

A series of Hfr crosses indicated that *mut-5* lies between the origins of HfrH and HfrC. To facilitate further mapping, strain KD1024 was converted into a good genetic donor by F'^{ts}*lac* integration (10). This donor, strain KD2002, was crossed with strain AA1158, yielding a Mut$^-$ recombinant, KD1067, which grew well and gave P1 transducing lysates of usably high titer.

When a series of markers was tested for co-transduction with *mut-5*, with strain KD1067 as the donor, it was found that the *mut-5-proA* co-transduction frequency was 21% (Table 2). *mut-5*, therefore, seemed to be a new mutator gene (23, 36), and was designated *mutD5*. Three of the other mutators isolated simultaneously with *mutD5* did not co-transduce with *proA* and, therefore, are not alleles of *mutD5*. The fourth, a weaker mutator, was not tested. We next found that *mutD5* co-transduced with *argF* (Table 3). The linkage is approximately 12%, even though in one cross, KD1069 *mutD5* × B90H *argF*, the apparent linkage is only 4%. We will discuss this datum later in the text, but we attribute this result to the fact that the donor in this cross contains two genes for ornithine transcarbamylase, *argI* and *argF*. Selection for Arg$^+$ in strain B90H does not discriminate between the two.

TABLE 1. *Bacteria and phage strains used*[a]

Strain	Source of derivation; reference	Sex	Relevant genotype and phenotype
Escherichia coli K-12 strains			
A23	C. Yanofsky; by UV from Y-mel (41)	F^+	*trpA23*
AB1157	C. Yanofsky (12)	F^-	*thr leu proA his argE strA*[b]
AA1158	This lab; P1 production of AB1157 to Spc^R Str^S	F^-	*thr leu proA his argE spcA*
AT713	A. Taylor (35)	?	*cysC39 argA8 lysA10 malA1 strA1* λ^R
C613	A. Pardee	F^-	*pyrB strA*
E36	A. Newton	F'^{ts} *lac*	F'^{ts} *lac*$^+$/MX74 (*lac pro*)$^\nabla$ Thi^-
ET3	V. Bryson (23)	?	*purA proA* (*thi*?)
KD1006	From AT713 by (i) transduction to Spc^R Str^S, (ii) spontaneous revertant to $Mal^+\lambda^S$	F^-	*cysC39 argA8 lysA10 spcA* λ^S
KD1024	From KD1006—see text	F^-	*mutD5 argA8 lysA10 spcA*
KD1051	From KD1024 by separate spontaneous mutations to (i) Str^R, (ii) Pro^-	F^-	*mutD5 argA8 lysA10 spcA strA* Pro^-
KD1057	Lac^- Pro^+ recombinant from X9030 × KD1051	F^-	*mutD5 lacZ X90 argA8 lysA10 spcA strA*
KD1067	Thr^+ Leu^+ Pro^+ recombinant from KD2002 × AA1158	F^-	*mutD5 his argE*
KD1069	From ET3 by (i) P1 (KD1067) × ET3 to Pro^+ *mutD5*; (ii) spontaneous reversion to Pur^+	F^-	*mutD5* Prototroph
KD1078	P1 (BD1001) × AA1158; Pro^+ selection	F^-	*mutD5 thr leu argE his spcA*
KD1079	ϕ80vir, *col* selection of *tonB-trpA, B* deletion in AA1158	F^-	*thr leu argE his proA* (*tonB-trpA, B*)$^\nabla$ *spcA*
KD1087	P1 (KD1078) × KD1079, Pro^+ selection	F^-	*mutD5* (*tonB-trpA, B*)$^\nabla$ *leu argE his spcA*
KD1088	From the same cross as KD1087	F^-	*mut*$^+$ rest as KD1087
KD1090	P1 (A23) × KD1088	F^-	*mut*$^+$ *trpA23 thr leu argE his spcA*
KD1096	P1 (A23) × KD1087	F^-	*mutD5 trpA23 thr leu argE his spcA*
KD1111	KD2007 × WW3352 $Azi^R Str^R$ selection	F^-	*mutD5 gal proA leu trp*$^\nabla$ Azi^R *strA*
KD1116	P1 (KD1096) × KD1079, $TrpB^+$ selection	F^-	*proA trpA23 thr leu argE his*
KD2002	Temperature-resistant Str^R, Lac^+ recombinant from E36 × KD1057	Integrated F'^{ts} *lac*	*mutD5* Lac^+ *lysA10 argA8 strA*
KD2003	P1 (ET3) × P4X, penicillin selection	Hfr (P4X type)	*proA metB1* λ
KD2007	Spontaneous Azi^R from KD2003	Hfr (P4X type)	*proA metB1* $Azi^R\lambda$
	A. Pardee (1, 35)	Hfr	*metB1* λ
PG13	This lab from KD1111 by successive P1 transductions to Trp^+, *mutD5*-Pro^+, Gal^+, Leu^+	F^-	*mutD5 strA*
W3350	T. H. Carter (5)	F^-	*su° strA*
WW3352	This lab from W3350: (i) P1 (W3110 Leu^-) × W3350, penicillin selection; (ii) *col*, ϕ80vir selection	F^-	*leu* (*trp tonB*)$^\nabla$ *strA su°*
X9030	A. Newton; Hfr3000 derivative (29)	HfrH	*lacZX90*
Escherichia coli B strains			
B90H	A. Pardee (25)	F^-	*argF argI his strA*
BD1001	P1 (KD1069) × B90H	F^-	*mutD5* Arg^+ *his strA*
DG336	A. Newton (39)	F^-	*his strA*
DG337	A. Newton (39)	F^-	*argA strA*
K-12 × B (Hfr × F^-) recombinant hybrids			
RD1001	KD2007 × DG336	F^-	*proA his strA* Azi^R
RD1002	KD2007 × DG337	F^-	*proA argA strA* Azi^R
Phage			
P1vir	A. Newton (18)		

[a] Symbols follow the conventions of Demerec et al. (11) and are defined by Taylor (34). Throughout the text, Mut^+ and *mut*$^+$ indicate the phenotype and the genotype, respectively, of low-mutation-rate, wild-type bacteria. The corresponding symbols for the conditional mutability phenotype and its genotype are Mut^- and *mutD5*, respectively.

[b] AB1157 is Thi^- and carries a nonsense suppressor. Most of its derivatives are untested for these characters but presumed to carry them.

TABLE 2. *Co-transduction of mutD5 and proA*

Donor	Recipient	Pro^+Mut^-/Pro^+	Frequency (%)
KD1067	ET3	16/98	16
KD1069	AA1158	23/100	23
KD1069	ET3	13/100	13
KD1069	KD2003	16/64	25
BD1001	AA1158	17/100	17
KD1078	KD1079	24/91	26
KD1087	RD1001	8/41	20
KD1087	RD1002	9/42	21
KD1087	KD1111	10/40	25
KD1096	KD1116	9/24	37

TABLE 3. *Co-transduction of mutD5 and argF*

Donor	Recipient	Arg^+Mut^-/Arg^+	Frequency (%)
KD1069	B90H	3/76	4
BD1001	B90H	22/188	12

Generality of mutD5 action. *mutD5* has been shown quantitatively to stimulate mutation of all *E. coli* genes with which it has been tested: resistance to NAL, DSM, RIF, and chlorate; reversion of the *leu*, *his*, and *argE* alleles of strain AB1157; and reversion of a large series of *trpA* alleles, including three frameshifts. Qualitatively, the same is true for mutation to azide and mutation at the *tfrA* locus. The mutator action of *mutD5* is thus general. Details of the mutation spectrum at the level of base substitution will be the subject of a later communication.

Conditionality of mutD5. The following experiment shows that *mutD5* activity is dependent upon growth conditions. Small, mutant-free inocula of 3,000 to 5,000 cells were distributed to multiple 25-ml flasks, one-half containing LB medium and one-half containing VB medium supplemented with 0.2% glycerol and the required amino acids. A similar series of flasks containing VB medium supplemented with 0.05% glycerol was done independently. Cultures were grown until saturated, and the frequency of mutants to Arg^+, Trp^+, His^+, Str^R, and Nal^R was determined (see Materials and Methods). It will be seen from the absolute (Table 4) or the relative mutation frequencies (Table 5) that LB medium caused the mutation frequency of all five markers to rise in the *mutD5* strain above the VB medium levels, and that the VB medium levels were higher in *mutD5* than in mut^+ strains. (For reasons not presently understood, *mutD5* reverts the *his* marker in VB medium at approximately the same frequency as does mut^+.) There is no medium-dependent change in the mut^+ mutation frequency. The medium-dependent phenotype of a *mutD5* strain is, however, reversible, since cultures grown to saturation in LB medium and plated on VB plates yield colonies which, when grown up in VB medium, mutate at rates characteristic of VB medium.

The conditionality of *mutD5* has also been shown quantitatively for mutation to Rif^R, Leu^+, and reversion of a large series of *trpA* alleles, including two frameshifts. Qualitatively, it has been shown for Azi^R and $TfrA^R$. No marker has yet been observed which is not conditionally mutated.

Thymidine dependence of mutD5 activity. One hypothesis to account for the medium dependence of the mutation rate is that some factor in LB medium promotes mutation. Preliminary experiments conducted by mixing the components of LB medium with VB medium ranked them as follows (descending mutational enhancement): tryptone, yeast extract, and NaCl (NaCl having no effect). A synthetic medium was developed, NM10, in which strain KD1096 grows as fast as in LB medium, yet does not increase its mutation rate (see Table 8). This was used as the basal medium in the following experiments so that variations in growth rate would not contribute to variations in mutation frequency. In NM10 medium, it was found that yeast extract increasingly stimulated mutability as the concentration was raised from 0.5 to 2.0%, although the maximal level was not equal to that in LB medium (data not shown). Therefore, 15 compounds not present in NM10 medium but likely to be in yeast extract were tested. The results were that only the deoxyribonucleosides, among the compounds tested, stimulated mutation. The free bases adenine, cytosine, thymine, and uracil (all present in NM10 medium at 50 μg/ml) did not stimulate mutation, nor did guanine (results not shown).

The mutational effector was studied further by inoculating supplemented NM10 medium with 10^6 cells per ml of strain KD1096 grown in VB medium. For a wide range of concentrations, neither 2-deoxyribose nor a mixture of four ribosides is mutagenic, whereas a mixture of five deoxyribosides is (Fig. 1). In separate experiments, it was also shown that each of the five deoxyribosides increased the mutant frequency when tested individually in our thymine-containing medium, NM10. For instance, Fig. 2 shows mutability as a function of the concentration of dThd and dAdo. Similar curves for dCyd, dGuo, and dUrd in the pres-

TABLE 4. *Mean mutation frequencies* $\times 10^{8a}$

Strain, medium, replicates	Mutant phenotype scored				
	Nal^R	Str^R	Arg^+	Trp^+	His^+
Strain KD1096 (*mutD5*)					
LB; 5	22,000 (±50%)	1200 (±50%)	11,000 (±67%)	2800 (±12%)	1200 (±68%)
VB (0.2% glycerol); 5	180 (±36%)	21 (±32%)	—[b]	38 (±29%)	—[b]
VB (0.05% glycerol); 8	140 (±41%)	—[c]	250 (±88%)	44 (±36%)	7.5 (±48%)
Strain KD1090 (*mut*$^+$)					
LB; 5	3 (±88%)	0.2 (±47%)	5 (±63%)[d]	0.2 (±17%)	0.7 (±140%)
VB (0.2% glycerol); 5	0.7 (±74%)	0.2 (±110%)	—[b]	0.1 (±200%)	—[b]
VB (0.05% glycerol); 4	0.3 (±200%)	—[c]	4 (±77%)	0.5 (±200%)	2 (±80%)

[a] Replicate cultures were inoculated with a small number of cells which had been grown in VB medium and contained no revertants. They were grown to saturation at 37 C. The mean mutation frequency is followed by ± one standard deviation expressed as percentage of the mean.

[b] All frequencies raised by selection for revertants in flask (limiting arginine or histidine).

[c] Not done.

[d] Mean of four cultures. Jackpot of 1.2×10^{-5} dropped.

TABLE 5. *Relative mutation frequencies*[a]

Genotype and medium	Mutant phenotype scored				
	Nal^R	Str^R	Arg^+	Trp^+	His^+
mutD5 in LB/*mutD5* in VB	**150**	**60**	**44**	**68**	**190**
mut$^+$ in LB/*mut*$^+$ in VB	6	1	1.2	0.7	0.3
mutD5 in VB/*mut*$^+$ in VB	400	100	60	100	3

[a] Calculated from the mean mutation frequencies of Table 4. Where two values are available in VB medium (e.g., for *mutD5*, Nal^R), their average has been used in the calculation.

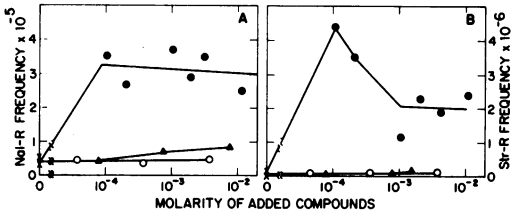

FIG. 1. *Stimulation of mutD5 mutation frequency by deoxyribose, ribonucleosides, and deoxyribonucleosides. Strain KD1096 (mutD5) from refrigerated stock culture was used to inoculate a series of cultures in NM10 medium at about* 10^6 *cells per ml. 2-Deoxyribose, a riboside mixture (adenosine, cytidine, guanosine, uridine, equal parts by weight), or a deoxyriboside mixture (deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine, deoxyuridine, equal parts by weight) was added to give the total concentrations specified. Cultures were grown to saturation at 37 C;* Nal^R *and* Str^R *frequencies were determined as described in Materials and Methods. (A)* Nal^R *frequencies; (B)* Str^R *frequencies. Symbols: ●, deoxyriboside mixture; ▲, riboside mixture; ○, 2-deoxyribose; ×, unsupplemented controls.*

ence of thymine are summarized in Table 6, which includes the dThd and dAdo data for comparison. The curve of mutant frequency versus dThd concentration, with a sharp maximum at 20 μM, is very similar to the dUrd curve; the dAdo curve, with a plateau from 0.2 to 2 mM, is similar to the dGuo curve. The dCyd curve differs from the others—it rises steadily, showing neither peak nor plateau, and ultimately reaches a mutational level approximating the other curves, but only at 2 mM. In the case of dAdo, this stimulation has been shown to be due to interaction with the thymine present in NM10 medium (see below; Fig. 3, Table 7). Deoxythymidine alone also stimulates mutation (see below; and Fig. 3). Deoxycytidine, deoxyguanosine, and deoxyuridine have not been tested in this way to test whether they are active in the absence of free thymine.

How does *mutD5* action depend on the concentration of dThd and dAdo? Exponential-phase cells of PG13 (*mutD5*) and KD1096 (*mutD5*) growing in VB medium containing 0.2% glucose at about 1.5×10^8 cells per ml were diluted 100-fold into the same medium prewarmed and supplemented with dThd or dAdo at various concentrations. For strain PG13, a prototroph, no further additions were made to the medium. For strain KD1096, the five required amino acids were supplied at 20 μg/ml. The determination of viable cell number on the zero, 0.1-μg/ml, and 100-μg/ml tubes for both series just after inoculation, and again 100 to 160 min later, showed that the cells continued to divide with, at most, a very short lag. Strain PG13 increased by an average factor of 1.8 (n =

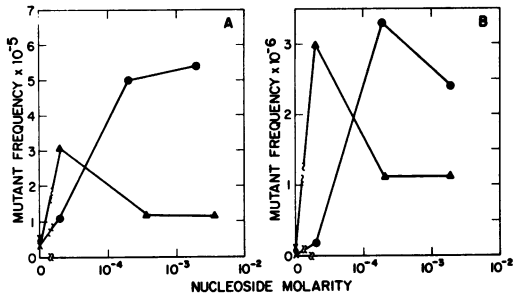

FIG. 2. *Stimulation of mutD5 activity by single deoxyribonucleosides in the presence of thymine. Strain KD1096 (mutD5) was used to inoculate a series of cultures in NM10 medium containing deoxyadenosine or thymidine in the amount specified. Details as in Fig. 1. This experiment was done simultaneously with that summarized by Fig. 1. Controls (zero addition) from Fig. 1. (A) Nal^R frequencies; (B) Str^R frequencies. Symbols: ●, deoxyadenosine; ▲, thymidine; ×, unsupplemented controls.*

TABLE 6. *Mutational response of KD1096 (mutD5) to deoxyribonucleosides in the presence of thymine*[a]

Additive	Maximal stimulation[b]	Concn causing maximum (mM)	Ratio of mutation in 2 mM additive to maximal mutation frequency[c]
dThd	7.0, 5.6	0.02, 0.02	0.38, 0.36
dUrd	11.4, 48	0.2, 0.02	0.51, 0.50
dAdo	12.1, 62	2, 0.2	1, 0.73
dGuo	28.5, 8.0	2, 0.2	1, 0.91
dCyd	7.8, 22	2, 2	1, 1

[a] Obtained from curves of Fig. 2 and similar curves for the other deoxyribonucleosides. Values from Nal^R curves are given first, separated by commas from corresponding values from Str^R curves.

[b] Mutation frequency in presence of additive divided by unsupplemented control values (each is the mean of 2): Nal^R, 4.5×10^{-6}; Str^R, 5.4×10^{-8}.

[c] Values close to 1.0 indicate small decline from peak; 1 indicates no decline.

5) in 100 min, and strain KD1096 increased by an average factor of 4.2 (n = 5) in 160 min. The mutation results are shown in Fig. 3. Clearly, dThd is capable of stimulating mutation, whereas dAdo is, at most, only slightly effective. The maximal dThd effect is found as low as 0.4 μM and, despite slight differences, in both strains the mutational responses to dThd pass through a maximum at 0.4 to 40 μM and fall off by about 50% at higher concentrations.

Knowing from this experiment that dAdo alone did not stimulate mutation, but that dAdo in the presence of other compounds and thymine did (Fig. 2A and B), we hypothesized that the cells were capable of forming sufficient dThd from thymine plus dAdo to cause mutation. Further experiments done in VB medium to compare the effects of thymine alone, dAdo alone, or both together support this hypothesis, for only the combination of thymine and dAdo is mutationally active (Table 7).

Inoculum effect. In the course of the experiments with dThd, an inoculum effect was noticed—heavy inocula from a saturated stock culture (inoculation with about 5×10^7 cells per ml) resulted in limited mutation, whereas smaller inocula (about 1,000 cells per ml) gave high mutation. The inoculum effect is also seen

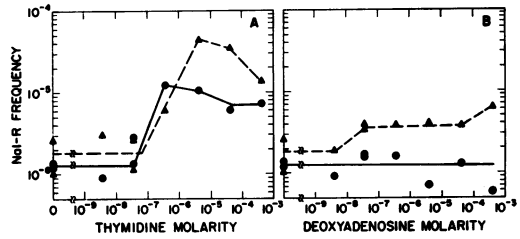

FIG. 3. *Effects of thymidine and of deoxyadenosine on mutD5 activity. For each curve one exponentially growing culture in VB medium was used to start a series of secondary cultures in VB medium (see text for details). The secondary cultures, containing thymidine or deoxyadenosine in the specified amounts, were grown to saturation and the Nal^R frequency determined. (A) Supplemented with thymidine; (B) supplemented with deoxyadenosine. Symbols: ▲, KD1096 (mutD5); ●, PG13 (mutD5).*

TABLE 7. *Interaction of thymine and deoxyadenosine to raise mutation level*[a]

Medium	Additions (100 μg/ml)	Strain KD1096 (*mutD5*) mutation frequency × 10^6 to:		Strain PG13 (*mutD5*) mutation frequency × 10^6 to Nal^R
		Nal^R	Str^R	
VB[b]	None	3.7	0.29	1.6
VB	dAdo	2.8	0.16	2.6
VB	Thy	7.0	0.26	0.9
VB	dAdo, Thy	64	4.5	49
LB	None	150	15	70

[a] Exponentially growing cells in VB medium were used to inoculate prewarmed medium at 1% by volume. Cultures were grown to saturation and titered. Each frequency is the mean of two independent cultures.

[b] For strain KD1096, VB medium contained 0.2% glycerol, 1 μg of thiamine-hydrochloride per ml, and 20 μg each of the required amino acids per ml. For strain PG13, VB medium contained 0.2% glucose and 1 μg thiamine-hydrochloride per ml.

to a smaller degree in LB medium cultures, presumably due to the same cause. It seems likely that this phenomenon can be explained by assuming that the inoculum degrades all available dThd before cell division begins (see Discussion).

Growth rate. The interaction, if any, of *mutD5* activity and growth rate was explored, since some of our results might be explained in terms of the varying growth rates experienced by cells in the several different media. The doubling times and mutation frequencies of two *mutD5* strains were, therefore, compared in four media under identical conditions at 37 C. Cultures were grown with aeration from small, exponential-phase inocula until they reached 2 $\times$ 10^8 to 4 $\times$ 10^8 cells per ml, at which time samples were plated for viable cells and mutant frequency. The rise in turbidity of each culture was determined periodically so that the doubling time was also known. Each medium was tested in duplicate in parallel flasks; mutation frequency and doubling time agreed closely between duplicates. The results with strain KD1096 (Table 8) show that mutation frequency and growth rate are independent—one may be high when the other is low and vice versa.

The results with strain PG13 confirm that slowly growing cultures can mutate at increased levels. In addition, however, and unlike strain KD1096, strain PG13 shows a high mutation frequency in rich medium lacking thymidine or its constituents (Table 8, row 3). This difference between strains presumably reflects an elevated internal pool of dThd or some active derivative in strain PG13 under these conditions.

DISCUSSION

Map position. Our average co-transduction frequency of 21% for *mutD5-proA* indicates a separation of approximately 0.8 min, using the available correlation data (35). Our best estimate of the *mutD5-argF* co-transduction frequency is 12% from the cross P1 (BD1001) $\times$ B90H in Table 3. The other cross presented there shows 4% linkage. We assume that this is approximately twofold too low since recent work shows that K-12 strains normally carry two unlinked genes for ornithine transcarbamylase, *argF* and *argI* (14), and *argI* co-transduces with *pyrB* (19). Thus, *argF* lies near 7 min and *argI* near 84 min. Either $argF^+$ or $argI^+$ alone is sufficient to convert a strain lacking ornithine transcarbamylase to Arg^+, but no *proA*-linked gene could be co-transduced with *argI*. The donor in cross 1 of Table 3 is a K-12 strain and, therefore, presumably $argF^+$ $argI^+$. Hence, approximately one-half of its Arg^+ progenies are expected to be due to the $argI^+$ gene, which is much too far away to be linked to *mutD5*. However, the donor in cross 2 of Table 3 is a Mut^- Arg^+ recombinant from the P1 (BD1001) $\times$ B90H cross, presumably of genotype *mutD5-*

TABLE 8. *Independence of doubling time (T_D) and mutation*[a]

Determination	T_D[b] (min)	Mutation frequency $\times$ 10^6 to:		Nal^R mutation frequency $\times$ 10^6
		Nal^R	Str^R	
Strain KD1096 (*mutD5*) grown on:				
VB glycerol + required amino acids	88	7.9	0.39	
VB glycerol + required amino acids + Thy + dAdo	84	91	8.2	
VB glucose + 20 amino acids + adenine	44	3.0	0.07	
LB	40	190	8.1	
Strain PG13 (*mutD5*)				
VB glycerol	90			1.8
VB glycerol + Thy + dAdo	106			15
VB glucose + 20 amino acids + adenine	60			13
LB	44			29

[a] From 10^6 to 10^7 exponentially growing cells in VB glycerol medium plus required amino acids were inoculated into 7.5 ml of prewarmed medium in side-arm flasks and grown with aeration at 37 C. Concentrations of medium constituents used in this experiment were: glycerol and glucose, 0.2%; required amino acids, 20 μg each/ml; thymine, adenine, and deoxyadenosine, 100 μg/ml; 20 L-amino acids, 50 μg each per ml. At about 50 Klett units (during exponential growth at 2 $\times$ 10^8 to 4 $\times$ 10^8 cells per ml), cultures were titered on LB, NAL, and DSM. Frequencies reported are the means of duplicate flasks, multiplied by 10^6.

[b] Determined graphically from turbidity measurements during exponential growth. Each is the mean of duplicate flasks.

argF$^+$ (*argI*$^+$) since our transduction procedures prevent double transduction events. Thus, we take the observed 12% Arg$^+$-Mut$^-$ frequency in cross 2 to be the true *argF-mutD5* co-transduction value. The tighter *proA-mutD5* linkage (21%) leads us tentatively to place *mutD5* counterclockwise of *proA* on the Taylor and Trotter map, giving the order *mutD5 proA argF lac*. This would put *mutD5* at approximately 5.7 min. (36). The order *proA argF lac* used here has recently been established as the correct one (14, 19), and represents a revision of the *argF* position on the 1970 map (34).

Conditional expression of the mutator gene. *mutD5* is a very strong mutator when the strain carrying it is grown in LB medium. In VB medium it is still comparable to known strong mutators. For rough comparison, we give the following Str^R frequencies, not from isogenic strains, but all measured in *E. coli* K-12: *mut*$^+$, 10^{-10} to 10^{-9}; *mutD5* in VB medium, 10^{-7}; *mutS3*, 10^{-7} (7); *mutT1*, 10^{-7} (9); *mutD5* in LB medium, 5×10^{-6} to 10^{-5}. Conditionality is a basic property of *mutD5*. It can be demonstrated both in cultures grown to saturation (Table 4) and in exponentially growing cells (Table 8). We have also investigated the kinetics of the rise in mutability and find that it begins to increase 20 min after a culture in VB medium is transferred to LB medium (Degnen and Cox, in preparation). The minimal time required for it to return to the basal level has not yet been determined, and at present we know only that during the time in which cells grown in LB medium form a colony on a VB medium plate, and then grow to saturation in VB liquid medium (about 72 h in all), the mutation rate has returned to typical VB medium levels. In addition, this conditionality has been demonstrated simultaneously for five independent markers (Tables 4 and 5), and all markers tested for conditional mutability exhibit it.

To account for the pronounced residual effect of *mutD5* in VB medium, several hypotheses are tenable. It may be due to an intrinsic defect in the structure or level of the *mutD5* gene product; to a very low level of an endogenously generated effector; to a trace of effector present in our media equivalent to less than 10^{-8} g of dThd (4×10^{-7} M) per ml, the threshold in Fig. 3A; or to the existence of two tightly linked additive mutator gene products, one conditional and very strong, the other constitutive and moderately strong. If a basal level mutator gene were segregating from the conditional high-mutating phenotype during the crosses used to construct our stocks, it might not have been detected by our spot test for mutation. Studies on the gene product currently underway in this laboratory may help elucidate this problem.

Existence of an effector for mutD5. Evidence has been presented showing that LB medium, NM10 medium plus various deoxyribonucleosides, and VB medium plus dThd or VB medium plus thymine and dAdo stimulate the mutability of *mutD5*. If we use the term effector in the general sense, embracing inducers and co-repressors as well as allosteric effectors (27), all of the conditions giving rise to enhanced mutability can be explained by a single effector derived from dThd. dThd exists in or can be synthesized directly from components in all media that stimulate mutation in strain KD1096. The ability of *E. coli* to make dThd from thymine plus dAdo via thymidine phosphorylase (EC 2.4.2.4) and purine nucleoside phosphorylase (EC 2.42.1) is well documented (3, 4, 20, 28), as is the ability of most other deoxyribonucleosides to serve as deoxyribosyl donors for the formation of dThd from thymine (20). LB medium contains yeast extract, a rich source of nucleic acids; and tryptone, which is also permissive for *mutD5* action, contains at least some, as judged by its ability to support growth of Thy$^-$ and Ade$^-$ strains.

Although it is clear that dThd serves to increase the mutability of *mutD5* strains, it is not clear whether dThd or a derivative of dThd is the active compound. The principal derivatives of dThd in *E. coli* are thymine; deoxyribose-1-phosphate; deoxyribose-5-phosphate; deoxythymidine mono-, di-, and triphosphate (dTMP, dTDP, and dTTP, respectively); dTDP-rhamnose; several other dTDP-sugars; and perhaps 2-deoxyribose. It seems to us likely that many of these compounds can be excluded as the possible effector, leaving as the most probable candidates dThd itself, dTMP, dTDP, or dTTP. The enzymatic reactions of *E. coli* important to this discussion are reviewed in Fig. 4.

Free thymine is easily excluded as the effector because it does not stimulate mutability when added to VB medium (Table 7), and it is not active in NM10 medium, which contains a wide variety of metabolites including thymine. Free exogenous 2-deoxyribose is excluded by the results in Fig. 1, as are deoxyribose-1-phosphate and deoxyribose-5-phosphate, since both compounds can be formed from dAdo as well as from dThd (Fig. 4).

The thymidine diphosphate sugars (dTDP-rhamnose, etc.), whose only established func-

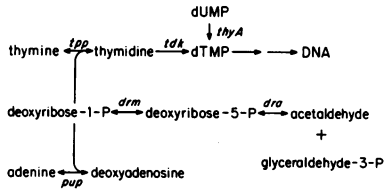

FIG. 4. *Portion of the E. coli metabolic map showing enzymes relevant to this paper. On the map, enzymes have been abbreviated by the corresponding E. coli genetic symbols, as follows: tpp, thymidine phosphorylase; pup, purine nucleoside phosphorylase; drm, phosphodeoxyribomutase; dra, deoxyriboaldolase; tdk, thymidine kinase; thyA, thymidylate synthetase.*

tion is as cell wall precursors in *Salmonella* (26, 30), seem unlikely candidates for the *mutD5* effector unless they are involved in a control loop normally serving to coordinate cell wall and DNA biosynthesis. There remain dTMP and dTDP as well as dThd itself. A choice among them may be possible by using strains blocked both in degrading dThd and in raising it to the nucleotide level. Such experiments with *mutD5* strains deficient in thymidine phosphorylase and thymidine kinase are currently being performed in this laboratory. The very low levels of dThd which bring about mutability suggest that the effector and its target molecule may be identified by equilibrium dialysis, gel filtration, or affinity chromatography.

The existence of an effector, either dThd or some derivative of it, also helps explain two other observations concerning *mutD5*. (i) The stronger stimulation of mutability by LB medium than by dThd or by dAdo plus thymine (Tables 4 and 7; and Fig. 3) probably is due to the competing destruction of dThd by thymidine phosphorylase. In *E. coli* this enzyme is present at a relatively high basal level (21) and is induced further by a breakdown product of dThd (2, 16). Moreover, phosphorolysis is inhibited in vivo by a large array of ribo- and deoxyribonucleosides, which range from complete to weak inhibitors. LB is a probable source of cytidine, deoxycytidine, adenosine, deoxyadenosine, uridine, and deoxyguanosine, all of which have been identified as inhibitors of dThd phosphorylase (4, 13). Deoxyadenosine may be playing a similar role in the dAdo plus thymine cultures of Table 6, as well as providing deoxyribosyl groups. (2) The inoculum effect reported here is probably also due to breakdown of dThd by thymidine phosphorylase. *mutD5* is inactive in the absence of DNA synthesis (Degnen and Cox, in preparation); and we think it not unlikely that with a heavy inoculum of lag phase cells, dThd is degraded to the inactive compounds thymine and deoxyribose-1-phosphate before the cells begin to grow (see reference 31).

Our results with LB medium could also be interpreted to mean that another effector is present, one that acts additively with dThd. However, the maximal stimulation seen when all deoxyribonucleosides are provided together in NM10 medium (Fig. 1) is approximately that found with dThd alone in VB medium (Fig. 3A), indicating that if another much stronger effector is present in LB medium, it is not a deoxyribonucleoside.

Independence of growth rate and mutD5 action. The shift in mutability of *mutD5* strains from low to high level can be totally decoupled from the growth rate of the cells. This independence, shown in Table 8 by strain KD1096, is evidence that enhanced mutability is not a result of any generalized physiological conditions occurring at a particular growth rate, such as increases in cell volume, cell mass, or the number of growing points per chromosome, over the range of doubling times 88 to 44 min. Note that we have employed two media of slow growth rate, with doubling times of 88 and 84 min, in which the mutation frequencies differ by factors of 12 to 21 depending on the presence or absence of dThd. Conversely, we have used two media of fast growth rate, with doubling times of 44 and 40 min, in which the mutation frequencies differ by approximately two orders of magnitude. And finally, in the medium having a doubling time of 44 min, the mutation frequencies are comparable to those in VB medium (actually ⅓ to ½ the levels found in the parallel control). Thus, in strain KD1096, the independence is complete. A similar experiment done with strain PG13 (also Table 8) confirms the ability of *mutD5* to mutate actively at slow growth rates (doubling time of 105 min). PG13 however, shows one difference: when growing faster (doubling time of 60 min) it mutates in the absence of any exogenous dThd source. In view of our other data, we attribute this result to an intrinsically higher cellular pool of the mutational effector in this strain. This is not unreasonable because of the extremely low amounts of dThd that are active in stimulating mutation (Fig. 3A). However, there are many known genetic differences between strains PG13 and KD1096, and doubtless innumerable unknown differences have also occurred between the time these strains diverged (5, 12) and the present. Therefore, it is impossible to discuss

what particular genetic differences between the strains causes this different mutational response to growth rate.

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