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The *mutT* Defect Does Not Elevate Chromosomal Fragmentation in *Escherichia coli* Because of the Surprisingly Low Levels of MutM/MutY-Recognized DNA Modifications[▽]

Ella Rotman and Andrei Kuzminov*

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois

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Nucleotide pool sanitizing enzymes Dut (dUTPase), RdgB (dITPase), and MutT (8-oxo-dGTPase) of *Escherichia coli* hydrolyze noncanonical DNA precursors to prevent incorporation of base analogs into DNA. Previous studies reported dramatic AT→CG mutagenesis in *mutT* mutants, suggesting a considerable density of 8-oxo-G in DNA that should cause frequent excision and chromosomal fragmentation, irreparable in the absence of RecBCD-catalyzed repair and similar to the lethality of *dut recBC* and *rdgB recBC* double mutants. In contrast, we found *mutT recBC* double mutants viable with no signs of chromosomal fragmentation. Overproduction of the MutM and MutY DNA glycosylases, both acting on DNA containing 8-oxo-G, still yields no lethality in *mutT recBC* double mutants. Plasmid DNA, extracted from *mutT mutM* double mutant cells and treated with MutM in vitro, shows no increased relaxation, indicating no additional 8-oxo-G modifications. Our Δ*mutT* allele elevates the AT→CG transversion rate 27,000-fold, consistent with published reports. However, the rate of AT→CG transversions in our *mutT*$^{+}$ progenitor strain is some two orders of magnitude lower than in previous studies, which lowers the absolute rate of mutagenesis in Δ*mutT* derivatives, translating into less than four 8-oxo-G modifications per genome equivalent, which is too low to cause the expected effects. Introduction of various additional mutations in the Δ*mutT* strain or treatment with oxidative agents failed to increase the mutagenesis even twofold. We conclude that, in contrast to the previous studies, there is not enough 8-oxo-G in the DNA of *mutT* mutants to cause elevated excision repair that would trigger chromosomal fragmentation.

To avoid mutagenesis, it is important to remove base analogs not only from DNA itself but also from the DNA precursor pools, for example, by hydrolyzing modified deoxynucleoside triphosphates. MutT of *Escherichia coli* is an example of such a nucleoside triphosphatase that in vitro hydrolyzes an oxidized form of dGTP that contains 8-oxo-G (7,8-dihydro-8-oxoguanine) (54) (Fig. 1A). The proofreading-deficient variants of both DNA polymerase III (Pol III; the replicative polymerase) and DNA Pol I (the main repair polymerase) of *E. coli* are capable of inserting this modified guanine with equal, but low, efficiency across cytosine or adenine (54, 68), with other DNA polymerases showing preferences one way or the other (22). In the 8-oxo-G · C scenario, both C and 8-oxo-G bases are in the normal *anti* conformation, whereas in the 8-oxo-G · A scenario, the 8-oxo-guanine assumes the *syn* conformation to pair with adenine, another purine (Fig. 1B) (55). If 8-oxo-G in *syn* conformation is incorporated opposite the A residue in the template strand but during the subsequent replication round flips back to the normal *anti* conformation to pair with the C residue, this leads to the AT→CG transversion (Fig. 1C, the bottom pathway). The *mutT* mutant of *E. coli* is specifically elevated for the AT→CG transversions and is one of the strongest mutators known, with a 10,000- to 50,000-fold increase over the wild type (17, 27, 78) (Fig. 1C, inset table). The MutT enzyme is the founding member of the big class of enzymes, called Nudix hydrolases (of which there are 13 in *E. coli* alone) that are proposed to target abnormal nucleotides, thus cleansing the nucleotide pools of the cell (6, 28).

8-Oxo-G can form not only in the DNA precursor pools but also in DNA itself, causing the GC→TA transversions (Fig. 1C, top pathway). *E. coli* is proposed to employ the MutM and MutY glycosylases to prevent this mutagenesis due to 8-oxo-G formation in DNA (57). MutM is responsible for removing 8-oxo-G across from cytosine (9) while MutY removes a misincorporated adenine across from an existing 8-oxo-G in the template strand (3, 58) (Fig. 1C, top pathway). MutY also wrongly attacks DNA when 8-oxo-G misincorporates across template A, judging by the decreased AT→CG mutagenesis in the *mutT mutY* double mutants relative to the *mutT* single mutants (27, 78) (Fig. 1C, inset table). As MutY removes the correct adenine in this case, it leaves the newly incorporated 8-oxo-guanine in the opposite strand to be later paired with cytosine, causing the transversion (Fig. 1C, bottom pathway).

8-Oxo-G is not the only base analog whose incorporation or formation within DNA is actively countered by specialized enzymes. In an analogous situation, the product of the *dut* gene in *E. coli* (31), a dUTPase (5, 30), hydrolyzes another noncanonical DNA precursor, dUTP, to prevent uracil incorporation into the replicating DNA in place of thymine (79). On the other hand, if uracil forms within DNA as a result of cytosine deamination (52), uracil DNA glycosylase (UDG), with the help of exonuclease III, acts to remove this base (50, 75). Interestingly, UDG does not distinguish between the U · A

* Corresponding author. Mailing address: Department of Microbiology, University of Illinois at Urbana-Champaign, B103 C&LSL, 601 South Goodwin Ave., Urbana IL 61801-3709. Phone: (217) 265-0329. Fax: (217) 244-6697. E-mail: kuzminov@life.uiuc.edu.

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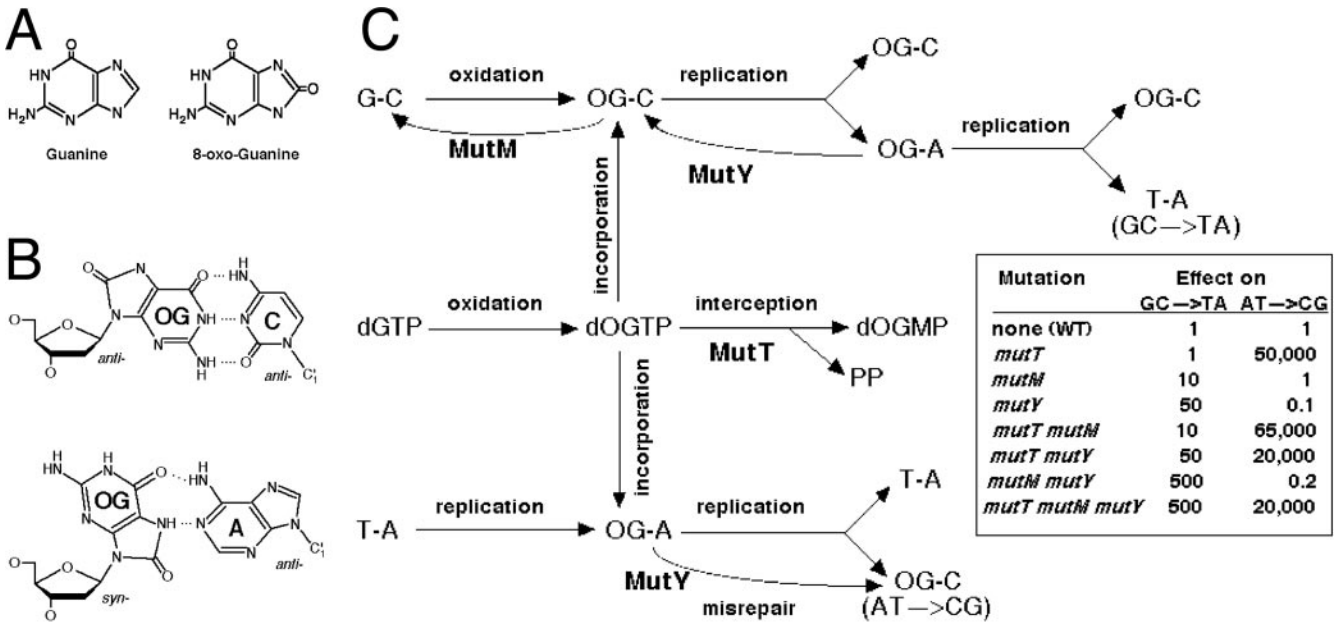

Mutation	Effect on GC—>TA	Effect on AT—>CG
none (WT)	1	1
mutT	1	50,000
mutM	10	1
mutY	50	0.1
mutT mutM	10	65,000
mutT mutY	50	20,000
mutM mutY	500	0.2
mutT mutM mutY	500	20,000

FIG. 1. 8-Oxo-guanine, its alternative pairing schemes, as well as the Mut proteins that counteract potential mutagenic consequences of 8-oxo-G (OG). (A) The structure of guanine compared to that of 8-oxo-guanine. (B) 8-Oxo-G · C pair (both in the *anti* conformation) versus 8-oxo-G · A pair (8-oxo-G is in the *syn* conformation). (C) The current understanding of the 8-oxo-G mutation-avoidance pathways (after references 27, 57, and 73). The top pathway leads toward the GC→TA transversion through A incorporation across template 8-oxo-G and is counteracted by MutM and MutY. Note that besides DNA-guanine oxidation, 8-oxo-guanine incorporation opposite the correct C residue without subsequent excision should also cause an increase in the GC→TA transversion down the line, as the template 8-oxo-G invites misincorporation of the A residue. The middle pathway shows 8-oxo-dGTP interception by MutT. The bottom pathway shows the AT→CG transversion through 8-oxo-G incorporation across the A residue in the template DNA and its enhancement by MutY. The table shows published data (27, 78) that were normalized to the wild type and rounded up to illustrate the logic of the scheme.

base pairs (the products of uracil incorporation) and the U · G base pairs (the products of cytosine deamination), excising both uracils equally well (51, 63), which leads to the elevated frequency of excision repair intermediates (nicks) in the *dut* mutants of *E. coli* (1, 43, 76).

Similarly, RdgB is the *E. coli* dITPase (10, 14, 15), whose function is to intercept the noncanonical DNA precursor dITP before hypoxanthine incorporates into DNA in place of guanine. If hypoxanthine forms within DNA by adenine deamination (36), endonuclease V initiates its removal (84, 85). Analogous to the action of UDG on DNA-uracils, endonuclease V fails to distinguish between H · C base pairs (incorporated hypoxanthine) and H · T base pairs (deaminated adenine) (84, 86), and this is postulated to lead to an increased frequency of excision repair in the *rdgB* mutants (10).

The high level of mutagenesis in *mutT* mutants translates into a high density of 8-oxo-G in their DNA that should trigger elevated levels of excision repair. The process of excision repair of a base analog is generally considered harmless to the cell. However, in both *dut* and *rdgB* mutants, whose chromosomal DNA undergoes more frequent excision repair, chromosomal fragmentation occurs (44). The removal of the modification leaves a nick in the DNA backbone that is efficiently repaired most of the time but is postulated to turn into a double-strand break if run over by a replication fork (46). Consequently, *dut* and *rdgB* mutants are nonviable in combination with defects in recombinational repair (10, 42), the only repair pathway to mend double-strand DNA breaks in *E. coli* (47). We reasoned that, in *mutT* mutants, the expected increase in MutM- and MutY-catalyzed excision around the incorporated 8-oxo-G should similarly result in replication fork collapse (Fig. 2A) and, therefore, in recombination dependence. Here, we describe our experiments to test this prediction.

MATERIALS AND METHODS

Growth conditions. Cells were grown in LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 250 μl of 4 M NaOH per liter) or on LB agar (LB medium supplemented with 15 g of agar per liter). M9 minimal plates contained 1× M9 salts, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ and were supplemented per liter with 10 mg of thiamine (B1), 15 g of agar, and 2 g of either glucose or lactose (61). Ampicillin (100 μg/ml), kanamycin (50 μg/ml), rifampin (25 or 100 μg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM), and riboflavin (50 μg/ml) were added when needed (all concentrations are final). All cultures were grown at 30°C with reciprocal shaking, unless otherwise stated.

Reagents. The Fpg (also named MutM) enzyme was purchased from New England Biolabs (NEB). Riboflavin, menadione, mitomycin C, and hydrogen peroxide were from Sigma. 8-Oxo-G was from Cayman Chemical. Methylene Blue was from Fisher Scientific. TE buffer contains 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Mutants. All *E. coli* strains used in these experiments were K-12, and most of them were derivatives of AB1157 (Table 1). Alleles were moved between strains by P1 transduction (61, 62). Precise deletion-replacement alleles of selected genes were created by the method of Datsenko and Wanner (20) and confirmed by PCR and phenotypic tests (except for the Δ*orf135* and Δ*dgt* mutants, for which no phenotypic tests are known). The Δ*mutS* and Δ*mutT* mutations were confirmed by increased formation of rifampin-resistant mutants (73). The Δ*ribA* mutants were confirmed by their requirement for riboflavin (37). Upon P1 transduction, the Δ*ribA* mutants formed large and small colonies; both required riboflavin and maintained their distinct size through subsequent restreaking. Since we did not know the reason for this difference, in subsequent tests we worked with only large colonies. The *recA* and *recBCD* mutants were confirmed

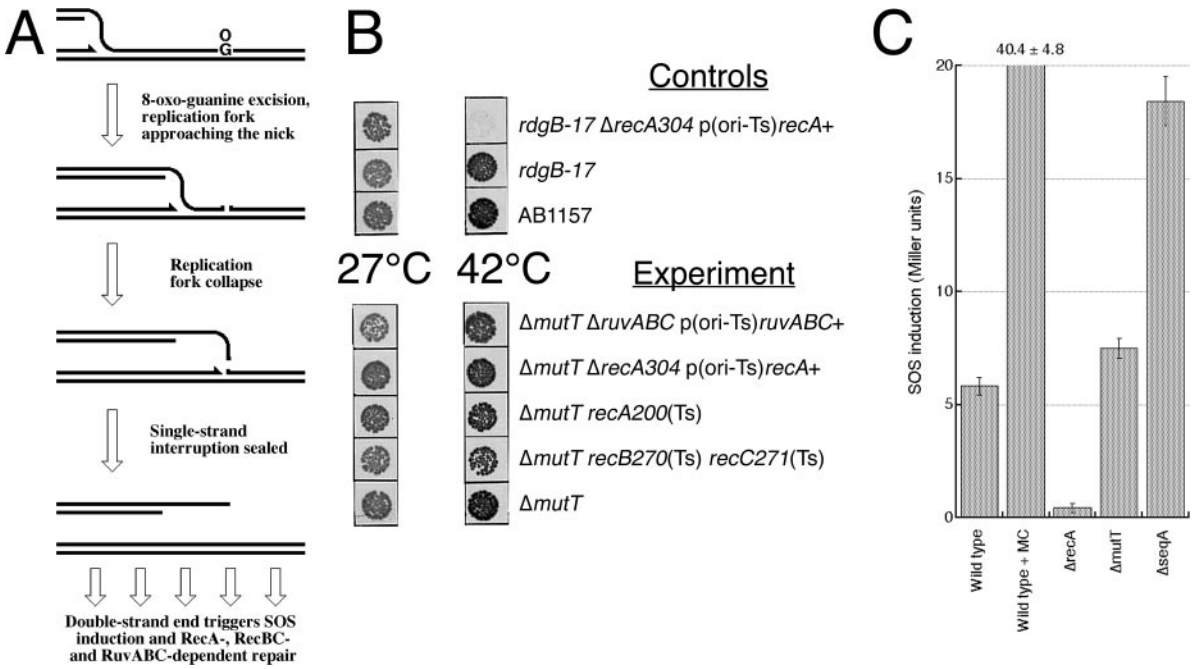

FIG. 2. Do the *mutT* mutants depend on double-strand break repair and induce SOS? (A) A scheme of the assumed replication fork collapse as a result of 8-oxo-guanine (OG) excision in the template DNA in front of the fork. The same logic applies for the *rdgB* mutants, only 8-oxo-guanine is replaced with hypoxanthine. (B) Viability of *mutT rec* double and triple mutants at the 42°C temperature, nonpermissive for the *rec* alleles. A total of 10 μl of 10^{-4} dilutions of rapidly growing cultures was spotted on LB plates and incubated either at 27°C for 36 h or at 42°C for 20 h. The Δ*recA304 rdgB-17* p(ori-Ts) *recA*$^+$ strain (44) is included as a positive control for the Rec dependence. The strains are as follows: AB1157, the wild-type strain; *rdgB-17* Δ*recA304*, EL002; *rdgB-17*, EL003; Δ*mutT* Δ*ruvABC*, ER6; Δ*mutT* Δ*recA304*, ER5; Δ*mutT recA200*(Ts), ER4; Δ*mutT recBC*(Ts), ER3; Δ*mutT*, ER2. (C) The level of SOS induction in Δ*mutT* mutant cultures. The *seqA* mutant is shown as an example of the level of SOS induction in RecA-dependent mutants (44). The strains are as follows: wild type, AK43; wild type+MC, AK43 grown in the presence of 100 ng/ml mitomycin C as a control for SOS induction (44); Δ*recA*, ER65; Δ*mutT*, ER27; Δ*seqA*, ER26. The values are means ± standard error of the mean (for *recA*, $n = 3$; for others, $n = 6$ to 10). The SOS level in the *mutT* mutant is significantly different from the one in wild-type cells (*t* test, $P_0 = 0.012$).

by their characteristic sensitivity to UV irradiation (80). Overproduction of MutM and MutY was confirmed by restoration of low titers of rifampin-resistant mutants in a *mutY* background (56).

Plasmids. The general features of the plasmids used are described in Table 2. pER5 is pMTL21 (13) into which a PCR-amplified fragment containing the *mutY* gene with its native promoter was cloned into the BamHI site codirectional with the *bla* gene. pER6 is pMTL21 into which a PCR-amplified fragment containing the *mutM* gene with its native promoter was cloned into the PstI site codirectional with the *bla* gene. The p*mutM* plasmid (82) is pTrc99A containing the *mutM* gene under the strong IPTG-inducible *trc* promoter. Plasmids pEAK2, pJSB2, pK96, pK134, pTrc99A, pX25A8L, and RP4 were described previously (Table 2).

Quantitative mutagenesis. To verify the mutator phenotypes, saturated cultures were serially diluted (10-fold at each step), and dilutions were spotted on LB plates (for titer) and on LB plates supplemented with 100 μg/ml rifampin (for mutants). The titer of resistant colonies was scored after 1 day of incubation at 37°C. To quantify *lac* reversion in the CC101 background, strains were propagated on M9-glucose minimal medium to maintain the episome, and 2- to 7-day-old colonies were inoculated in LB broth. For CC101, 10 ml of a saturated culture was concentrated and spread on M9-lactose minimal medium (12), while the Δ*mutT* mutants had 10 μl of each dilution spotted on the plate. Since the small amount of glucose present in lactose preparations allowed formation of Lac$^-$ microcolonies, cell titer was determined by counting these microcolonies on M9-lactose plates using a stereomicroscope. The counts of total cells obtained this way were identical to counts obtained by regular plating on M9-glucose plates. To determine the effect of mutagenic treatments on the reversion frequency, colonies of CC101 and CC101 Δ*mutT* were inoculated into LB medium and grown to an optical density at 600 nm (OD_{600}) of ~0.01, and the culture was split, with one-half receiving treatment while the other served as a control. Both halves were then grown for another 15 to 17 h before being plated on M9-lactose. Lac$^+$ colonies of CC101 were counted after 48 h, while Lac$^+$ CC101 Δ*mutT* colonies were counted after 24 h under the microscope because the strain exhibited adaptive-like mutagenesis (26) at later times.

Spot test for synthetic lethality. Growth at the nonpermissive temperature was first assayed by diluting an overnight culture 100-fold, growing it to 5×10^8 cells/ml, diluting 0.2 μl in 5 ml of 1% NaCl, and spotting by 10-μl amounts. Plates were incubated at either 27 to 30°C [permissive temperature for *rec*(Ts) alleles] or 42°C (the nonpermissive temperature). Colonies were given approximately 24 h to grow at both 42°C and at 30°C. Since preliminary results indicated that the viability of saturated cultures was similar to that of rapidly growing cultures, in subsequent assays we spotted 10 μl of a 10^{-6} dilution of a saturated culture.

SOS induction. To determine the level of SOS induction in the cell, Δ*mutT*::*kan* was P1 transduced into AK43 (44), a strain containing a MuΔX::*cat*-derived construct with the *lacZ* gene fused under the *sfiA* promoter (66). When the cells are under SOS-induced stress, the promoter is expressed, and the level of β-galactosidase can be quantitatively measured by the modified protocol of Miller (61), using 200 μl of culture (44). As a positive control, wild-type cells containing the *sfiA*::*lacZ* fusion were treated with 100 ng/ml mitomycin C, a cross-linking agent. As a control for no SOS induction we used Δ*recA* cells.

Pulsed-field gel electrophoresis. The pulsed-field gel electrophoresis protocol was adapted from reference 44. Overnight LB cultures were diluted to an OD_{600} of 0.02 in LB medium and grown in the presence of 2.5 to 10 μCi of [^{32}P]orthophosphoric acid to an OD_{600} of 0.35 (approximately 4 to 5 h) at 30°C for Δ*recBCD* strains or for 1 h at 22° and 3 h at 37° (to OD_{600} values of 0.6 to 0.9) for *recBC*(Ts) strains. All cultures were then brought to an OD_{600} of 0.35. Cells from 0.5- to 1-ml aliquots were spun down, washed in 1 ml of TE buffer, and resuspended in 60 μl of TE buffer. Five microliters of proteinase K (10 mg/ml) and 65 μl of 1.2% agarose in lysis buffer (see below) were added and mixed by pipetting. A total of 110 μl of the mixture was then pipetted into the plug mold and allowed to solidify. The plugs were incubated overnight at 60°C in the lysis buffer (1% sarcosine, 50 mM Tris-HCl, and 25 mM EDTA). Samples were

TABLE 1. Bacterial strains

Strain source and name	Relevant genotype or description[a]	Reference or derivation
Strains from previous studies		
AB1157[b]	Rec^+	4
AK43	*sfiA*::*lacZ* (MuΔX *cat*)	44
AK105	*dut-1*	42
CC101[c]	*lacZ* E461stop	18
CC104[c]	*lacZ* E461A	18
CM1319[d]	*mutM103*::mini-Tn*10*	CGSC 7738; 11
JC9941	*recA200*(Ts)	A. J. Clark
JC10287	Δ(*srlR-recA*)*304*	19
JB1	Δ*recBCD3*::*kan*	62
JJC754	Δ*ruvABC*::*cat*	60
L-41[e]	DH5α $recA^+$ Δ*mltB*::*cat*	42
SK129	*recB270*(Ts) *recC271*(Ts)	45
Strains from the present study		
BB034	Δ*dgt-10*::*cat*	Deletion-replacement
EL003	*rdgB-17*	Kan_{pRL27} insertion
EL002	Δ*recA304 rdgB-17*	JC10287 × P1 EL003
ER2	Δ*mutT9*::*kan*	Deletion-replacement
ER3	Δ*mutT9*::*kan recB270*(Ts) *recC271*(Ts)	SK129 × P1 ER2
ER4	Δ*mutT9*::*kan recA200*(Ts)	JC9941 × P1 ER2
ER5	Δ*mutT9*::*kan* Δ*recA304*	JC10287[f] × P1 ER2
ER6	Δ*mutT9*::*kan* Δ*ruvABC*	JJC754 × P1 ER2
ER8	Δ*recBCD4*	JB1; *kan* removed by pCP20
ER11	DH5α $recA^+$ *mutM103*::mini-Tn*10*	L-41 × P1 CM1319
ER12	*mutM103*::mini-Tn*10*	AB1157 × P1 ER11
ER13	Δ*mutT9*::*kan mutM103*::mini-Tn*10*	ER2 × P1 ER12
ER14	Δ*mutT9*::*kan* Δ*recBCD4*	ER8[g] × P1 ER2
ER15	Δ*seqA20*::*kan*	Deletion-replacement
ER16	Δ*seqA21*	ER15; *kan* removed by pCP20
ER26	*sfiA*::*lacZ* Δ*seqA20*::*kan*	AK43 × P1 ER15
ER27	*sfiA*::*lacZ* Δ*mutT9*::*kan*	AK43 × P1 ER2
ER46	Δ*seqA20*::*kan recBC*(Ts)	SK129 × P1 ER15
ER48	Δ*mutT10*	ER9, *kan* removed by pCP20
ER49	Δ*ribA*::*cat*	Deletion-replacement
ER50	Δ*mutT9*::*kan* Δ*ribA*::*cat*	ER2 × P1 ER49
ER51	Δ*mutT9*::*kan* Δ*ribA*::*cat recA*(Ts)	ER4 × P1 ER49
ER52	Δ*orf135*::*kan*	Deletion-replacement
ER53	Δ*mutT10* Δ*orf135*::*kan*	ER48 × P1 ER52
ER54	Δ*mutT10 recA*(Ts)	ER4; *kan* removed by pCP20
ER55	Δ*mutT10* Δ*orf135*::*kan recA*(Ts)	ER54 × P1 ER52
ER56	Δ*mutT10* Δ*ribA*::*cat* Δ*orf135*::*kan recA*(Ts)	ER54 × P1 ER49
ER57[c]	CC101 Δ*mutT9*:: *kan*	CC101 × P1 ER2
ER58[c]	CC104 Δ*mutT9*::*kan*	CC104 × P1 ER2
ER59[c]	CC101 Δ*mutT10*	ER57; *kan* removed by pCP20
ER60[c]	CC101 Δ*mutT10* Δ*orf135*::*kan*	ER59 × P1 ER52
ER61[c]	CC101 Δ*ribA*::*cat*	CC101 × P1 ER49
ER62[c]	CC101 Δ*mutT9*::*kan* Δ*ribA*::*cat*	ER57 × P1 ER49
ER63	Δ*mutT10* Δ*ribA*::*cat* Δ*orf135*::*kan*	ER53 × P1 ER49
ER64[c]	CC101 Δ*orf135*::*kan*	CC101 × P1 ER52
ER65	*sfiA*::*lacZ* Δ*recA304*	JC10287[f] × P1 AK43
ER66[c]	CC101 Δ*dgt-10*::*cat*	CC101 × P1 BB034
ER67[c]	CC101 Δ*mutS234*::*cat*	CC101 × P1 LA24
ER68[c]	CC101 Δ*mutT9*::*kan* Δ*dgt-10*::*cat*	ER57 × P1 BB034
ER69[c]	CC101 Δ*mutT9*::*kan* Δ*mutS234*::*cat*	ER57 × P1 LA24
LA24	Δ*mutS234*::*cat*	Deletion-replacement

[a] All strains have the AB1157 background unless indicated otherwise (see below).

[b] The complete genotype includes the following: F^- λ^- *rac- thi-1 hisG4* Δ(*gpt-proA*)*62 argE3 thr-1 leuB6 kdgK51 rfbD1 araC14 lacY1 galK2 xylA5 mtl-1 tsx-33 glnV44 rpsL31*.

[c] The complete genotype included in addition the following: *ara* Δ(*lac proB*)XIII F *lacI-Z-proB*$^+$.

[d] This is a B/r (not K-12) strain. Other mutations include the following: F^- *sulA1 trpE65*(Oc).

[e] A complete genotype includes in addition the following: F′ *endA1 hsdR17*(r^- m^+) *glnV44 thi-1 recA1 relA1* Δ(*lacIZYA-argF*)*U169 deoR*(ϕ80-Δ*lacZM15*) *gyrA*(NalR).

[f] The *recA* defect was complemented with the pEAK2 plasmid for the purpose of P1 transduction. The plasmid was cured at 37°C once the construct was confirmed.

[g] The *recBC* defect was complemented with the pK134 plasmid for the purpose of P1 transduction. The plasmid was cured at 37°C once the construct was confirmed.

TABLE 2. Plasmids

Plasmid source and name	Description (ori, drug resistance, other genes)	Reference or derivation
Previous studies		
pEAK2	pSC101(Ts), *bla*, *recA*	42
pJSB2	pSC101(Ts), *bla*, *ruvABC*	10
pK96	pSC101, *aad*, *recC ptr recB recD*	49
pK134	pSC101(Ts), *bla*, *recC ptr recB*	62
pMTL21	pMTL, *bla*	13
pMutM	pBR322, *bla*, *mutM*	82
pTrc99A	pBR322, *bla*	2
RP4	IncPα, *bla tet aph*	67
pX25A8L	pBR322, *bla*, *Neurospora crassa* DNA	41
This study		
pER5	pMTL, *bla*, *mutY*	pMTL21::*mutY*
pER6	pMTL, *bla*, *mutM*	pMTL21::*mutM*

loaded into a 1.0% agarose gel in 0.5× Tris-borate-EDTA buffer and run at 6.5 V/cm with a pulse time of 90 s for 7 h, 105 s for 8 h, and 125 s for 8 h in a Gene Navigator (Pharmacia) instrument. The gel was vacuum dried onto a piece of chromatography paper (Fisher) for 2 h at 80°C and then exposed to a PhosphorImager screen until signals from the wells reached between 300,000 and 900,000 counts.

Quantification of in vivo nicking. Cultures of AB1157, Δ*mutT*, and *dut-1* strains, containing plasmid pX25A8L, pK96, or RP4, respectively, were grown to an OD_{600} of 0.6 to 0.7. A total of 450 μl of the culture was mixed with 50 μl of 10% sodium dodecyl sulfate, and the total DNA was extracted with 500 μl of phenol, then with 500 μl of phenol:chloroform, and finally with 500 μl of chloroform, followed by two ethanol precipitations (48). Then, 250 ng (pK96), or 500 ng (pX25A8L), or 1 μg (RP4) of the DNA preparation was run on a 1.1% gel at 2.5 V/cm for 20 h. The DNA was transferred to a hybridization membrane (Amersham) by capillary transfer and probed with ^{32}P-labeled plasmid DNA (random hexamer labeling; NEB kit).

Treatment with Fpg (MutM) in vitro. Plasmid DNA from 40 ml of saturated culture and 120 ml of log phase culture (OD_{600} of 0.8) of strains harboring plasmid RP4 was prepared by the alkaline lysis procedure (8). A total of 150 ng of this DNA was treated with 0.08 U of the MutM DNA glycosylase (the Fpg protein; NEB) in a 100-μl reaction mixture using 1× NEB buffer 1 (10 mM bis-Tris propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol [pH 7.0 at 25°C]) at 37°C for 5 to 10 min. Negative controls were treated with the buffer only. The reaction was stopped with 10 μl of 10% sodium dodecyl sulfate, and DNA was ethanol precipitated prior to loading on a 1.1% gel, which was run for 20 h at 2.5 V/cm. The DNA was transferred to Hybond N^+ membrane (Amersham) by capillary transfer and probed with ^{32}P-labeled plasmid. To induce 8-oxo-G in DNA in vitro, DNA solutions were supplemented with 10 to 100 μM methylene blue and illuminated by a 60-W bulb for 15 min at a distance of 15 cm (25). Two consecutive ethanol precipitations removed the bulk of methylene blue before the gel electrophoresis.

RESULTS

The *mutT* and *recA* mutations do not show synthetic interactions. MutT was the first recognized example of a DNA precursor pool sanitizer (7), the prototype for the class of functions to which Dut and RdgB also belong (28). Since both *dut* and *rdgB* mutants show dependence on recombinational repair for viability (10, 42), we tested *mutT* mutants for a similar requirement. The *mutT* gene was deleted and replaced with a kanamycin resistance cassette. The allele was P1 transduced into strains carrying conditional alleles of *recA*, *recBC*, or *ruvABC*, either temperature-sensitive alleles or complete deletions complemented with functional genes on *ori* temperature-sensitive plasmids. In these strains, a mutant dependent on recombinational repair is able to grow at 30°C but is nonviable or severely weakened at 42°C (10, 42). We found that, in contrast to *dut* or *rdgB* mutations, the *mutT* mutation did not confer synthetic lethality or even inhibition at 42°C in recombinational repair-deficient strains (Fig. 2B). This suggests that, in contrast to the *dut* or *rdgB* mutants, the level of DNA modifications and/or excision repair in *mutT* mutants is not high enough to cause such a dramatic phenotype as dependence on recombinational repair. We decided to detect the elevated level of DNA modification or excision repair by more sensitive assays, measuring SOS induction, chromosomal fragmentation, or accumulation of MutM-recognized DNA modifications.

***The mutT* mutant is slightly induced for the SOS response.** Recombinational repair mends chromosomal damage—a unique class of DNA damage that disables the whole chromosome. Whenever cells deploy recombinational repair, they also initiate the SOS response, boosting the cell's capacity to repair and tolerate DNA damage, while at the same time inhibiting cell division to allow more time for repair. The SOS response increases the expression of some 30 genes and decreases the expression of approximately 20 more genes (16, 24). The RecA protein senses the damage or disruption in replication and stimulates self-cleavage of the LexA repressor, thereby allowing transcription of LexA-repressed genes (53). The level of SOS induction can be measured by placing a reporter gene downstream of a LexA-inducible promoter. If the level of SOS induction in *recA* mutants is taken for the background of the procedure, there is a measurable SOS induction in wild-type cells, reflecting the ongoing repair of continuously generated chromosomal lesions (Fig. 2C). All known RecA-dependent mutants show various degrees of additional SOS over the wild-type level (44), consistent with the detectable additional chromosomal damage. Even such RecA-independent mutants as *rnhA*, *rep*, and *uvrD* also induce SOS (38, 66, 77), so *mutT* mutants, even though shown to be Rec independent, might still induce some SOS. Indeed, when we introduced the Δ*mutT* mutation into the SOS indicator strain, we detected a small statistically significant SOS induction over the wild-type level (Fig. 2C), suggesting a possibility of additional chromosomal damage in *mutT* mutants.

***The mutT* mutation does not increase chromosomal fragmentation.** An even more sensitive assay for elevated levels of excision in chromosomal DNA is the direct detection of chromosomal fragmentation in pulsed-field gels. Chromosomal fragmentation reflects formation of double-strand DNA breaks as a result of excision repair in replicating DNA (43). The *recA* and *recBC* mutants, deficient in recombinational repair, cannot mend these double-strand breaks and, consequently, accumulate subchromosomal fragments. In addition, *recBC* mutants are also deficient in the major linear DNA degradation activity (exonuclease V) (47), the absence of which makes linear chromosomal DNA stable in these mutants. These DNA pieces of several hundred kilobase pairs in length can be separated from the intact chromosome by pulsed-field gel electrophoresis. During pulsed-field gel electrophoresis, intact (circular) chromosomes remain in the wells, while linear subchromosomal pieces migrate into the lane (59). The fraction of the total DNA in the lanes is measured, and a

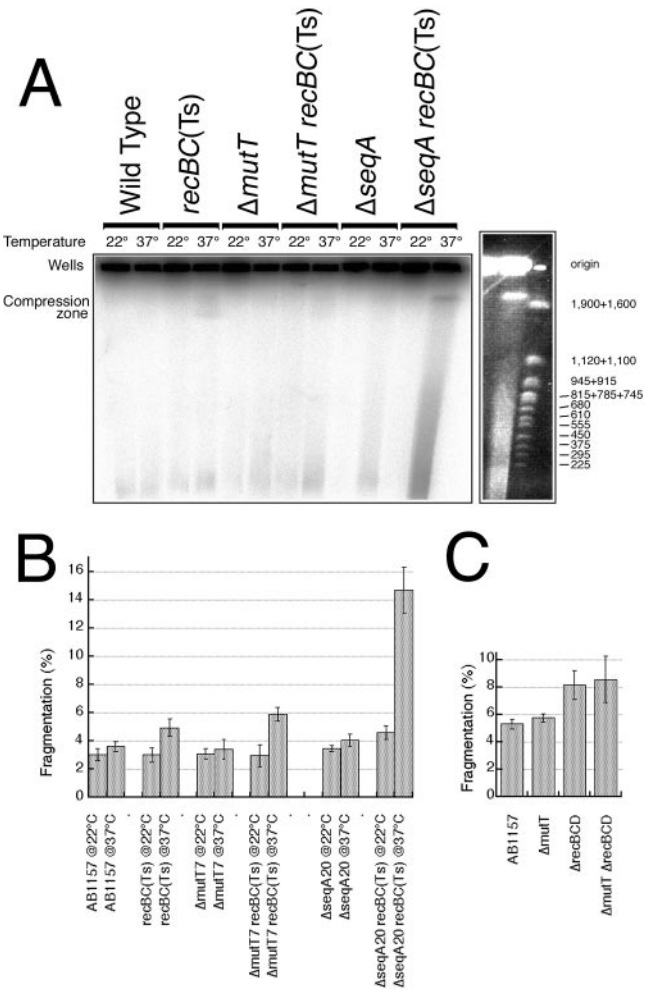

FIG. 3. The level of chromosomal fragmentation in the *mutT* mutants. The *seqA* mutants are shown as an example of a significant chromosomal fragmentation (44). (A) A representative pulsed-field gel of the chromosomal DNA isolated in agarose plugs from the indicated strains, grown at the indicated temperatures. To the right, the last two lanes after ethidium bromide staining show their relation to molecular weight markers (yeast chromosomes; size indicated in kbp). Strains are the following: wild type, AB1157; *recBC*(Ts), SK129; Δ*mutT*, ER2; Δ*mutT recBC*(Ts), ER3; Δ*seqA*, ER15; Δ*seqA recBC*(Ts), ER46. (B) The level of chromosomal fragmentation, averaged from three independent experiments run on different days. The strains are the same as in panel A. The data are means ± standard error of the mean ($n = 3$). The level of fragmentation in Δ*mutT recBC*(Ts) at 37°C is not significantly different from that in *recBC*(Ts) at 37°C (*t* test, $P_0 = 0.12$). (C) The level of chromosomal fragmentation in the Δ*mutT* Δ*recBCD* mutant, averaged from seven independent experiments run on different days. The data are means ± standard error of the mean. The strains are the following: wild type, AB1157; Δ*mutT*, ER2; Δ*recBCD*, ER8; Δ*mutT* Δ*recBCD*, ER14.

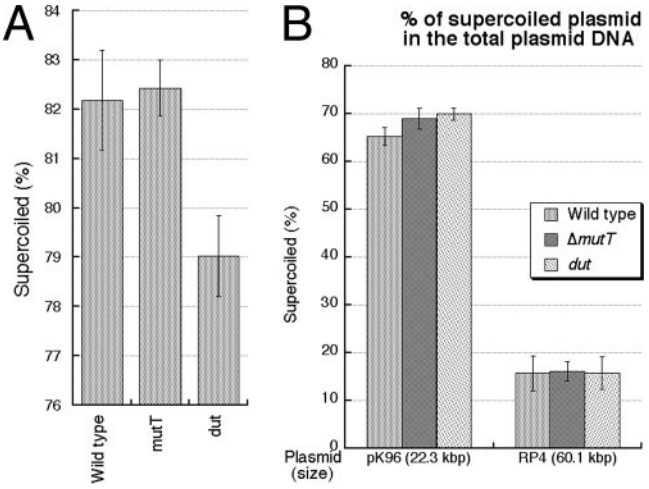

FIG. 4. The level of in vivo plasmid relaxation in a *mutT* mutant. (A) Total plasmid DNA of pX25A8L (30.0 kbp) was extracted from wild-type (AB1157), Δ*mutT* mutant (ER2), and *dut-1* mutant (AK105) strains as described previously (43) and run on a 1.1% agarose, and the fraction of supercoiled plasmid species in the total plasmid DNA was determined after blot hybridization with plasmid-specific probes. The data points are means ± standard error of the mean ($n = 11$). The *dut* value is significantly different from the wild-type value (*t* test, $P_0 = 0.025$). (B) The procedure described in panel A was performed with plasmids pK96 and RP4. The data points are means ± standard error of the mean ($4 \leq n \leq 10$). The plasmid size is shown in parentheses. The differences in the levels of supercoiling likely reflect the different replicons.

greater percentage indicates a higher level of fragmentation. Single mutants of *dut* and *rdgB* do not show excessive chromosomal fragmentation, but the double mutants *dut recBC*(Ts) and *rdgB recBC*(Ts) accumulate 20 to 25% of the total DNA in subchromosomal fragments after 4 h of incubation at 37°C, the nonpermissive temperature for the *recBC*(Ts) allele (44). In contrast, a Δ*mutT recBC*(Ts) double mutant does not show an increase in fragmentation over a *recBC* single mutant at 37°C (Fig. 3A and B), and the double mutant Δ*mutT* Δ*recBCD* does not show an increase over the level of a Δ*recBCD* single mutant (Fig. 3C). The absence of chromosomal fragmentation in the *mutT recBC* double mutants is consistent with the findings that the *mutT rec* double mutants are not synthetically lethal, and *mutT* single mutants are only slightly induced for the SOS response.

The *mutT* mutation does not increase the level of excision. Next, we considered the possibility that base analogs are incorporated into the DNA of *mutT* mutants and are then excised, but this excision is somehow different from the excision in *dut* or *rdgB* mutants in that it does not cause chromosomal fragmentation. In *dut* mutant strains, the high level of uracil misincorporation combined with the efficient excision repair elevates the steady-state level of nicks in DNA (1, 43, 76). Nicks can be detected as relaxation of supercoiled plasmids. Total DNA, extracted from wild-type and mutant cells, contains both supercoiled and relaxed plasmid forms. A mutant with a high level of nicks will show more relaxed plasmid species compared to the wild-type cells. To detect possible plasmid relaxation due to 8-oxo-G excision in *mutT* mutants, we used a 30-kbp plasmid that we had successfully used before with *dut* mutants (43). We found no significant difference in the levels of relaxed plasmid between *mutT* mutants and wild-type cells (Fig. 4), indicating no increase in excision repair due to the *mutT* defect. The level of detection in this assay with this number of repetitions (Fig. 4A) is six additional relaxation events per genome equivalent (the statistically significant difference in supercoiling of 3.8% $[(82/79 - 1) \times 100]$ translates into 1 relaxation event per 1.58×10^6 nucleotides [60,000

nucleotides in the plasmid divided by 0.038], which, with almost 9.3×10^6 nucleotides in the *E. coli* genome equivalent, gives ~6).

When we carried out the same assay in two other big plasmids, 22.3 kbp and 60.1 kbp long, driven by different replicons, we again found no difference between the wild type and the *mutT* mutant strain, but this time the *dut* mutant was also no different from the wild type (Fig. 4B), suggesting some replicon-dependent variations in the sensitivity of the assay. The lack of increased relaxation in *mutT* mutants can be due either to the low level of 8-oxo-G incorporation or perhaps to an inefficient action of MutM and MutY glycosylases in vivo. For example, endonuclease V, which initiates excision of hypoxanthine from DNA, is known to act slowly and to be swamped by increased hypoxanthine incorporation in *rdgB* mutants (10).

The *mutT* and *recA* mutations do not show synthetic lethality even when MutM and MutY proteins are overproduced. To test the possibility that MutM and MutY reactions in vivo are slow, we increased the copy number of either MutM or MutY proteins in *mutT* mutant cells by placing the functional *mutM* and *mutY* genes on a high-copy-number vector. The *mutT rec* strains, transformed with the resulting plasmids, were still viable at 42°C, although all strains were slightly weakened by the high copy number of $mutY^+$ or $mutM^+$ genes (Fig. 5A). The *recBC* mutants were affected more than rec^+ cells or *recA* mutants (Fig. 5A; also data not shown). Even though the constructed plasmids complemented the mutator phenotype of the *mutY* mutant, there was a possibility that the *mutM* and *mutY* genes are downregulated under their native promoters, negating our attempt to overproduce them. However, a plasmid expressing the *mutM* gene under the control of a strong inducible promoter (82) gave essentially the same results: all *recA* and *recBCD* mutants showed inhibition in response to *mutM* induction, regardless of the presence or absence of the *mutT* deletion (Fig. 5B).

Upon closer examination, we noticed that the original Δ*mutT recBC*(Ts) transformants with the $mutM^+$ multicopy plasmid pER6 were heterogeneous, giving rise to stable clones that showed various degrees and patterns of temperature sensitivity (not shown). The four major patterns were (i) no sensitivity, (ii) slower growth and no loss of titer, (iii) normal growth and loss of titer, and (iv) slow growth and loss of titer. Some heterogeneity was also noticed for Δ*mutT* single mutants (Fig. 5A). Since these patterns were stable and all transformants were confirmed for all three expected phenotypes—*mutT* (increased mutagenesis), *recBC* (UV sensitivity), and MutM overproduction (decreased mutagenesis in *mutY* mutants)—at face value this result suggests that MutM overproduction is detrimental in *mutT recBC* conditions, but some additional unknown factors are at play. However, our general conclusion is that overproduction of DNA glycosylases acting on 8-oxo-G-containing DNA does not create a problem for the *mutT* mutant cells, suggesting little additional 8-oxo-G in the DNA of *mutT* mutants.

No additional MutM-recognized DNA modifications in DNA from *mutT mutM* double mutants. To assess the level of MutM-recognizable modifications in the DNA of *mutT* mutants directly, we measured the in vitro relaxation of the supercoiled plasmid by purified MutM (Fpg). The MutM glycosylase has an associated DNA lyase activity that, after removal of the modified base, also nicks the DNA backbone at the generated abasic site (64). Thus, removal of a single base modification by MutM converts a supercoiled plasmid into its relaxed form. As a control for the MutM activity and specificity, we treated pure supercoiled plasmid DNA in vitro with 10 μM methylene blue in the presence of light to specifically generate 8-oxo-G in this DNA (25, 72) and observed an almost complete plasmid relaxation upon MutM treatment (Fig. 6A). It should be pointed out that the treatment combining methylene blue and light by itself breaks DNA strands (72), which can be seen as a slight increase of relaxed species without the enzyme treatment (Fig. 6A).

The in vitro MutM-nicking assay for 8-oxo-G was performed once before, using a 4.4-kbp plasmid isolated from *mutT*, *mutT mutM*, and *mutT mutY* mutant cells, and no MutM-recognized DNA modifications were found (81). To maximize the sensitivity of the assay, we used the 60.1-kbp plasmid RP4. When isolated by the alkaline lysis procedure, approximately 60% of the plasmid is in the supercoiled form, while after the treat-

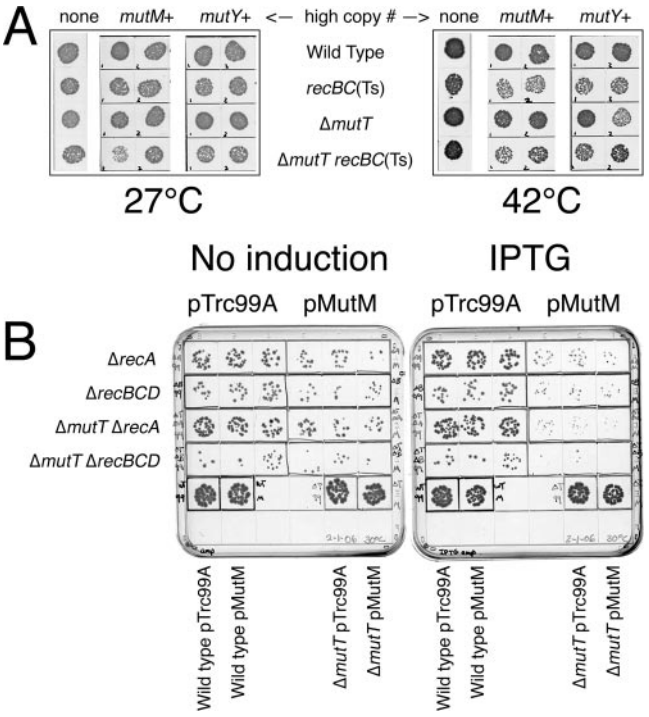

FIG. 5. High copy number of $mutM^+$ and $mutY^+$ does not kill *mutT rec* mutants. (A) The effect of high-copy-number plasmids carrying the $mutM^+$ or $mutY^+$ genes on the viability of *mutT* and *recBC* mutant strains. A total of 10 μl of 10^{-4} dilutions of rapidly growing cultures was spotted on LB plates and incubated either at 27°C for 36 h or at 42°C for 20 h. Strains are as follows: wild type, AB1157; *recBC*(Ts), SK129; Δ*mutT*, ER2; Δ*mutT recBC*(Ts), ER3. Plasmids are the following: $mutM^+$, pER6; $mutY^+$, pER5. (B) No *mutT rec* synthetic lethality even when MutM is overexpressed. A total of 10 μl of 10^{-6} dilutions of saturated cultures was spotted on LB plates either supplemented with IPTG to induce $mutM^+$ expression or left untreated (no induction) and incubated at 30°C for 24 h. The left three spots on each plate are three independent cultures carrying the pTrc99A vector plasmid; the right three spots are three independent cultures carrying the overexpression pMutM plasmid. The strains are the following: wild type, AB1157; Δ*mutT*, ER2; Δ*recA*, JC10287; Δ*recBCD*, ER8; Δ*mutT* Δ*recA*, ER5; Δ*mutT* Δ*recBCD*, ER14.

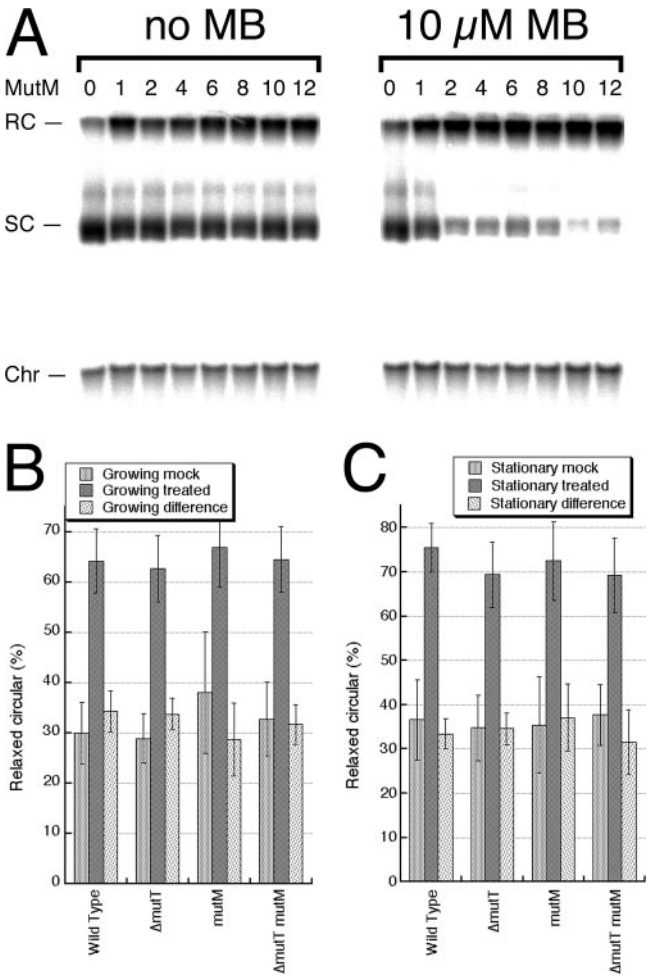

FIG. 6. The level of MutM-recognized DNA modifications in plasmid DNA. (A) RP4 DNA (positive control), treated in vitro with methylene blue (MB) and light, can be subsequently nicked in vitro with the MutM (Fpg) enzyme, indicating the presence of 8-oxo-guanine residues. Amount of MutM is expressed in 10^2 units, so that, for example, 8 corresponds to 0.08 units. RC, relaxed circular plasmid DNA; SC, supercoiled plasmid DNA; Chr, chromosomal DNA. (B) The level of relaxation caused by MutM-recognized DNA modifications in plasmid DNA isolated from growing cultures of wild-type (AB1157), Δ*mutT* (ER2), *mutM* (ER12), and Δ*mutT mutM* (ER13) strains. Mock, plasmid incubated in the buffer only; treated, plasmid incubated in the complete reaction mixture; difference, the result of the subtraction of the mock value from the treated value. The data points are means of five independent measurements done on different days ± standard error of the mean. (C) The level of relaxation caused by MutM-recognized DNA modifications in plasmid DNA isolated from stationary cultures of the same strains.

ment with MutM, only 30% of the plasmid still remains supercoiled (Fig. 6B and C). Plasmid DNA was extracted from wild-type cells and *mutT* and *mutM* single mutants, as well as from *mutT mutM* double mutants, from both stationary and exponentially growing cells (Fig. 6B and C). Stationary cells should have time to remove lesions before the DNA extraction, but the log-phase cells would contain unexcised 8-oxo-G. Plasmid preparations were treated with MutM or mock treated with buffer alone and run in agarose gels for subsequent blot hybridization. We expected to see an increase in relaxed species for the *mutT mutM* double mutant, which should incorporate elevated levels of 8-oxo-G but would be unable to remove them.

We found that although the plasmid from both stationary and growing cells was significantly relaxed by the enzyme treatment, the degree of relaxation was independent of the *mutT* or *mutM* status of the strains from which the plasmid DNA was purified (Fig. 6B and C). The lowest standard error in these experiments was ~10% of the mean, allowing us to reliably detect a ~30% difference in the degree of relaxation between the two sets of data (which gives a zero class calculated as $1 - 0.3 = 0.7$). Since the frequency of zero class permits calculation of the average number of events per molecule using the Poisson distribution, $-\ln(0.7)$ is 0.36. Dividing the 122,000 nucleotides of RP4 by this frequency of events per molecule gives one event per $\sim 3.4 \times 10^5$ nucleotides, or fewer than 27 MutM-recognizable DNA modifications per *E. coli* genome equivalent ($\sim 9.3 \times 10^6$ nucleotides); this is the sensitivity limit of our current measurements with this number of repetitions. On the basis of this in vitro MutM relaxation study, we conclude that, compared with the wild-type cells, there are fewer than 27 additional MutM-recognized DNA modifications per genome of *mutT* mutants.

The expected high AT→CG transversion mutagenesis in our Δ*mutT* mutants. At this point we had to address the possibility that our Δ*mutT* allele is an atypical one in that it is not highly mutagenic. Although a qualitative rifampin test confirmed the mutator phenotype of our Δ*mutT* mutants, the increase was modest in the quantitative rifampin test (Fig. 7A), as expected for the *rpoB* mutagenic target that detects all types of base substitutions (29). To quantify the highly specific mutator phenotype of the Δ*mutT* allele, we introduced it into CC101 and CC104 indicator strains (18). The *E. coli* CC101 strain is incapable of growing on lactose as the sole carbon source due to a point mutation in the *lacZ* gene, but reverts to Lac^+ via AT→CG transversion, the type of mutation specifically induced in *mutT* mutants (83). We found that our Δ*mutT* allele elevates the spontaneous Lac^+ reversion frequency in the CC101 strain more than 10^4-fold (Fig. 7B), which is consistent with the published values (27, 78). The mutagenesis is highly specific, because the Δ*mutT* allele does not increase reversion to Lac^+ in CC104, an otherwise isogenic strain that detects GC→TA transversions (not shown). These findings indicate that our Δ*mutT* allele induces a high level of DNA modification, which causes the expected high level of mutagenesis yet somehow does not translate into chromosomal breakage.

The density of 8-oxo-G is still too low for chromosomal fragmentation. The high level of specific mutagenesis in the *mutT* mutant suggests a considerable density of the modified guanine base in the DNA of *mutT* mutants. Remarkably, since 8-oxo-G is so mutagenic, pairing equally well with C and with A, the actual density of 8-oxo-G in DNA can be assessed from the mutation rates per cell per generation. Such calculations using the values reported for the same CC101 *mutT* construct in previous studies (27, 78) give from 54 to 560 8-oxo-G modifications per genome equivalent (Table 3). Excision repair of this level of DNA modification should cause a considerable chromosomal fragmentation. However, our values for the rate of AT→CG transversions in the CC101 *mutT* strain translate into only 3.0 to 4.4 8-oxo-G modifications per genome equiv-

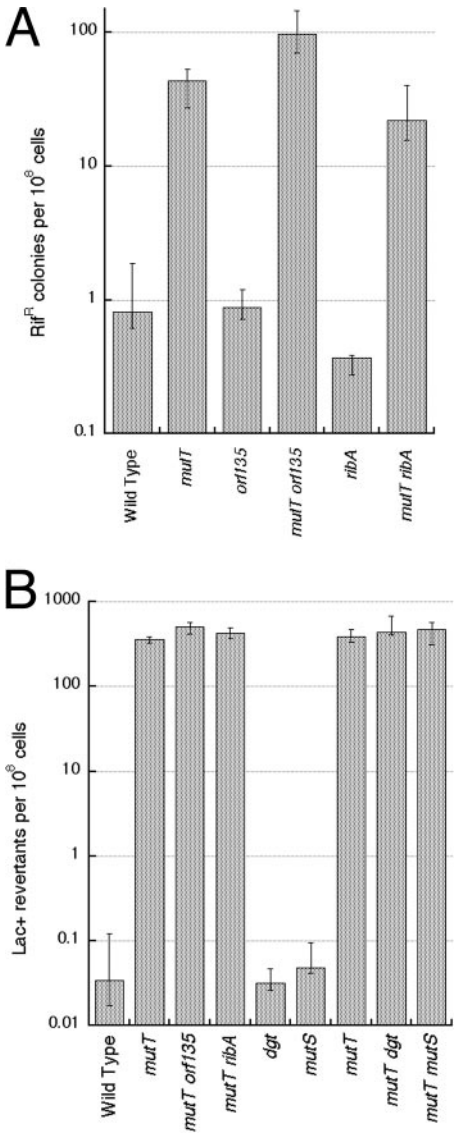

FIG. 7. Mutagenesis tests of *mutT* mutants and their derivatives. (A) Rifampin test. (B) Lac$^+$ reversion in CC101 (a specific test for AT→CG transversion). Values are medians ± first and third quartiles (n = 4 to 19).

alent. The main reason is that while the mutagenicity of our *mutT* allele is the same as the normalized mutation rates used in the previous studies (Table 3), the mutation rate in the CC101 strain is 22 to 125 times lower. It should be noted that a similarly low level of mutagenesis was already reported for this CC101 strain in an independent study from this laboratory (12).

The calculated level of three to four 8-oxo-G modifications in the DNA of *mutT* mutants is 10 times lower than the steady-state level of endonuclease V-recognized modifications in the DNA of *rdgB* mutants (about 42 per genome equivalent) (10) and is even lower than the steady-state level of uracil excision events in *dut* mutants (16 per genome equivalent) (43) (Table 4). We would not be able to detect this level of modification in *mutT* mutants using our most sensitive assay that measures

TABLE 3. Mutation rates in CC101 versus CC101 *mutT* strains for the AT→CG transversions and the calculated densities of 8-oxo-G in the chromosomal DNA of CC101 *mutT* strains

Study[a]	Mutation rate per cell generation[b]			Density of 8-oxo-G (no. of modifications)[c]
	μ of CC101 (10^{-11})	μ of CC101 *mutT* (10^{-6})	Relative increase (n-fold)	
1	134	23	~17,200	54
2	750	240	32,000	560
This study	4.9–6.9	1.3–1.9	~27,000	3.0–4.4

[a] Study 1, Vidmar and Cupples (78); study 2, = Fowler et al. (27).
[b] To calculate mutation rates for studies 1 and 2, the frequencies that these studies report were treated as medians (21). Mutation rates (μ values) for all three studies were calculated by the method of Lea and Coulson, as explained by Rosche and Foster (70). In addition, mutation rates for this study were also calculated by the method of Drake or by the P_0 method, again as explained by Rosche and Foster (70). Therefore, a range is reported instead of a single value.
[c] The density of 8-oxo-G per genome equivalent in *mutT* DNA was calculated by multiplying the obtained mutation rates per cell per generation at this single site by the size of the *E. coli* genome (4.64×10^6), dividing by 4 (since only A residues, which are 25% of all bases in the *E. coli* DNA, can mutate into C residues) and multiplying by 2 (since 8-oxo-G pairs equally well with C [no mutation] and with A [the detected transversion].

plasmid relaxation by Fpg treatment in vitro because it is below the level of its sensitivity (about 27 modifications per genome equivalent) (Table 4). It is also unlikely that excision of these infrequent modifications can cause detectable chromosomal fragmentation.

Attempts to further increase 8-oxo-G incorporation in *mutT* mutants. (i) H_2O_2, menadione and 8-oxo-G. To increase the level of 8-oxo-G incorporation into DNA artificially, we used the *mutT* derivative of CC101 to seek conditions that would further increase the level of AT→CG transversions. Since 8-oxo-G is the product of guanine oxidation and the two most common reactive oxygen species are hydrogen peroxide and

TABLE 4. Comparison of the density of DNA modifications and the steady-state level of relaxation events in mutants affecting either interception of noncanonical DNA precursors or excision of noncanonical DNA bases or both

System name[a]	Mutant	Density of modifications (no.)[b]	Steady-state level of relaxation events (no.)[c]
Dut+Ung	*dut*	<22*	16.3
	ung	286	NA
	dut ung	174,000	NA
RdgB+Nfi	*rdgB*	42	ND
	nfi	<2*	NA
	rdgB nfi	42	NA
MutT+MutM	*mutT*	<27*	<6
	mutM	<27*	NA
	mutT mutM	<27*	NA

[a] For Dut+Ung, all data are from Kouzminova and Kuziminov (43), except for data for *dut ung*, which are averaged from Warner et al. (79). For RdgB+Nfi, all data are from Bradshaw and Kuzminov (10). Data for MutT+MutM are from the present study.
[b] All values are densities per genome equivalent. Density of modifications was measured by following relaxation of a big plasmid DNA in vitro with a DNA repair enzyme specific for this modification (as shown, for example, in Fig. 6). Values marked with an asterisk are equivalent to wild-type levels.
[c] Steady-state level of relaxation events per genome equivalent was measured by assaying for plasmid relaxation in vivo (as shown, for example, in Fig. 4). NA, not applicable; ND, not done.

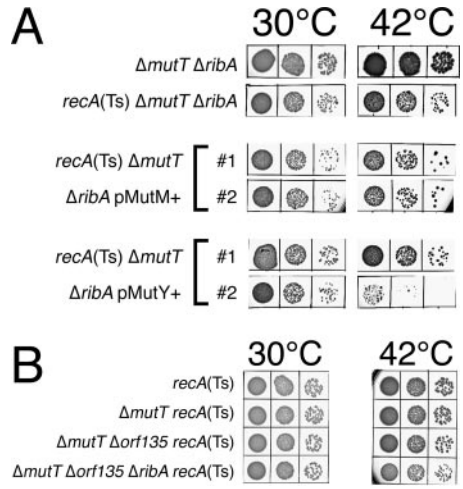

FIG. 8. High copy numbers of *mutM*$^+$ and *mutY*$^+$, as well as other potential defects in 8-oxo-dGTP interception, do not kill *mutT rec* mutants. (A) The effect of high-copy-number plasmids carrying the *mutM*$^+$ or *mutY*$^+$ genes on the viability of the *recA*(Ts) *mutT ribA* triple mutant (ER51). A total of 10 μl of serial dilutions of saturated cultures was spotted on LB plates and incubated at either 30°C or 42°C for 24 h. pMutM$^+$, pER6 plasmid; pMutY$^+$, pER5 plasmid. (B) The *recA200*(Ts) *mutT ribA orf135* quadruple mutant is viable. Ten microliters of serial dilutions of saturated cultures was spotted on LB plates and incubated at either 30°C or 42°C for 24 h. Strains are the following: *recA*(Ts), JC9941; Δ*mutT recA*(Ts), ER4; Δ*mutT* Δ*orf135 recA*(Ts), ER54; Δ*mutT* Δ*orf135* Δ*ribA recA*(Ts), ER56.

superoxide (32), we treated CC strains with subinhibitory concentrations of hydrogen peroxide (300 μm) and the superoxide-generating chemical menadione (400 μm) but did not find any increased mutagenesis (not shown). We also sought to increase mutagenesis in the CC101 Δ*mutT* strain by growing it in the presence of 10 mM concentrations of exogenous 8-oxo-G, but this did not increase the number of AT→CG transversions either (not shown).

(ii) *ribA*. Although the AT→CG transversion mutagenesis is dramatically increased in *mutT* mutants (Fig. 7B), our failure to further increase it with various treatments can be rationalized by the existence of other activities in the cell specifically preventing 8-oxo-G incorporation into DNA. One of the proposed back-up enzymes for 8-oxo-dGTP interception is RibA, encoding GTP-cyclohydrolase II, the enzyme catalyzing the first step in riboflavin biosynthesis that converts dGTP into pyrophosphate, formate, and 2,5-diamino-6-(ribosylamino)-4-(3H)-pyrimidinone 5′-phosphate (37). The proposed intermediate of this reaction resembles 8-oxo-G, and the RibA enzyme was shown to have an 8-oxo-dGTPase activity in vitro, while the *mutT ribA* double mutant was claimed to exhibit an increased level of Rif^r mutagenesis (37), implying that the RibA protein can partially fulfill MutT's role in cleansing the nucleotide pools of 8-oxo-dGTP. We deleted the *ribA* gene from the chromosome but found that the *mutT ribA* double mutant had the same level of Rif^r mutagenesis as the *mutT* single mutant (Fig. 7A), whereas the *mutT ribA recA* triple mutant was still viable (Fig. 8A). When the MutM and MutY proteins were overexpressed, the triple mutant *mutT ribA recA* cells were viable but lost titer (Fig. 8A), suggesting that the *ribA* defect does increase slightly the level of MutM- and MutY-recognized DNA modifications.

(iii) *orf135*. *orf135* (also termed YnjG and NudG) is another Nudix hydrolase with reported activity against 8-oxo-dGTP (35). Although the oxidized dGTP does not seem to be its primary substrate (65), the level of Rif^r mutagenesis of an *orf135* mutant is claimed to be elevated twofold (34), suggesting possible overlap of function between the MutT and Orf135 enzymes. We deleted *orf135* from the chromosome to generate a Δ*mutT* Δ*orf135* double mutant and a Δ*mutT* Δ*ribA* Δ*orf135* triple mutant. We tested the mutation frequency (either to Lac$^+$ or Rif^r phenotypes) of the single and double deletion mutants in CC101 and found no increase in the specific AT→CG transversions (Fig. 7B) but a threefold increase in Rif^r mutagenesis in the *mutT orf135* double mutant (Fig. 7A). We also tested for *recA* dependence of the Δ*mutT* Δ*ribA* Δ*orf135* mutant and found that the quadruple mutant *mutT ribA orf135 recA* grew at 42°C almost as well as the double and the triple mutants (Fig. 8B), indicating the absence of a significant increase in excision of modified bases.

(iv) *dgt*. One more possible backup enzyme is dGTP-triphosphohydrolase (the product of the *dgt* gene) (69), the enzyme identified by Kornberg and colleagues as a dGTP-hydrolyzing contamination in early preparations of DNA Pol I (40). Since the only known substrate of the enzyme in vitro is dGTP, there is a suspicion that the real substrate in vivo is a modified dGTP, with 8-oxo-dGTP being a possible candidate. We inactivated the *dgt* gene by constructing a precise deletion in the chromosome but found that the Δ*dgt* mutation increases the level of AT→CG transversions in neither wild-type nor *mutT* mutant backgrounds (Fig. 7B).

(v) *mutS*. It can be calculated from the published *lacI* spontaneous mutagenesis data that the mismatch repair defect in *E. coli* (due to the *mutH* or *mutL* or *mutS* mutation) significantly increases the frequency of both GC→TA transversions (87 times) and AT→CG transversions (18 times) (71). Although data from one locus may not be representative, they are suggestive nonetheless, so we introduced Δ*mutS* mutation into both CC101 and CC101 Δ*mutT* strains. However, we found that the original AT→CG transversion mutagenesis was not significantly affected in either case (Fig. 7B).

DISCUSSION

We tested whether a Δ*mutT* mutant showed dependence on double-strand break repair due to a postulated incorporation and excision of 8-oxo-G. The extremely high reported rates of AT→CG transversions in *mutT* mutants suggested significant 8-oxo-dGTP incorporation, resulting in a high steady-state level of 8-oxo-G in their DNA and elevated excision. Similar situations in *dut* and *rdgB* mutants lead to chromosomal fragmentation and dependence on recombinational repair (10, 42, 44). To our surprise, we found no synthetic interactions between the *mutT* defect, on the one hand, and *recA*, *recBCD*, or *ruvABC* mutations on the other. It should be noted that the *mutT1 recA* mutants were reported to be viable (17); however, this is the first time that the viability of *mutT recBC* mutants was tested. For example, the *rep* and *rnhA* mutants are synthetically lethal with the *recBC* defect but viable with the *recA* defect (33, 77). On the other hand, the synthetically lethal *dut*

recA mutants were for a long time considered viable (23, 39), apparently due to a rapid accumulation of suppressors (42). Finally, our study was the first one to employ a complete *mutT* deletion allele, which could have behaved differently from the *mutT1* allele. Three additional assays corroborated the observed independence of the *mutT* mutants of recombinational repair. In the first, we used pulsed-field gel electrophoresis to detect chromosomal fragmentation, and by that test, the *mutT* mutant did not exhibit a level significantly above the wild type. In the second test, we measured SOS induction, a sensitive indicator of DNA damage. All RecA-dependent mutants show SOS induction (44), and even some RecA-independent mutants do (38, 66, 77). The *mutT* mutant showed little SOS induction, in agreement with the lack of chromosomal fragmentation. In the third assay, we measured the level of relaxed plasmid DNA in vivo due to MutM and MutY DNA-glycosylase activity. A large plasmid extracted from the *mutT* mutant showed the same ratio of supercoiled-to-relaxed DNA as the plasmids from wild-type cells. We conclude that the absence of MutT does not cause increased excision repair and, therefore, does not translate into elevated chromosomal fragmentation.

Since we assumed that our *mutT* mutants have the reported high absolute levels of mutagenesis (27, 78), we next considered the possibility that the 8-oxo-G-triggered excision by MutM and MutY is inefficient. If so, overproduction of the MutM and MutY glycosylases should elevate the excision and may cause chromosomal fragmentation requiring the recombinational repair system. However, while *recA* and *recBCD* single mutants did show some inhibition with the extra glycosylases, the result was largely independent of the *mutT* status of the strains. Moreover, when we extracted plasmid DNA from *mutT* and *mutM* mutants and treated it in vitro with a pure MutM enzyme, we found that the level of MutM-recognizable lesions was the same in DNA from all backgrounds, indicating that the *mutT* defect does not detectably increase the level of MutM-recognized DNA modifications. At this point we measured the rate of AT→CG transversions in our wild-type and *mutT* mutant strains to compare them with the reported data. We found that, whereas the increase in mutagenesis due to the *mutT* defect matches the values reported in the previous studies, the absolute level of AT→CG transversions is up to two orders of magnitude lower because of the much lower basal level in our wild-type strain (which is confirmed in an independent study [12]). We have noticed an adaptive-like mutagenesis (26) in the CC101 strain, about a twofold increase in the number of Lac^+ colonies between day 2 and day 8 (Fig. 9), but its magnitude is not enough to account for the difference in results from different laboratories. Although we still do not know the reason for the disparate levels of mutation rates, the reduced absolute level of AT→CG transversions in our *mutT* mutants translates into only three to four 8-oxo-G modifications per genome equivalent, which is not enough to cause chromosomal fragmentation of the type observed in the *dut* and *rdgB* mutants (10, 42, 44). These results generally agree with the earlier studies that were unable to detect either 8-oxo-dGTP in extracts of *E. coli* cells (74) or 8-oxo-G in the DNA from *mutT mutM* and *mutT mutY* double mutants (81).

Using the CC101 indicator strain, we tried to find conditions that would further increase the AT→CG transversions in *mutT* mutants. In particular, we removed RibA and Orf135, two proposed back-up enzymes for MutT, as well as the Dgt dGTPase, a suspected noncanonical nucleotide interceptor. In our hands, these additional mutations failed to increase specific mutagenesis in the *mutT*-deficient background. Moreover, the *mutT orf135* and *mutT ribA* double mutants were both viable in combination with the *recA* defect, as was the *mutT ribA orf135 recA* quadruple mutant. Inactivation of the methyl-directed mismatch repair system with a *mutS* deletion also failed to increase the level of AT→CG transversions in *mutT* mutants. We also tried oxidizing conditions and direct 8-oxo-guanine supplementation but without success. We conclude that 8-oxo-G incorporation in the DNA of *mutT* mutants is at the maximal level but does not translate into chromosomal fragmentation because the absolute levels of 8-oxo-G in DNA still remain low. Therefore, the *mutT* case turned out to be an exception that proves the rule that the high density of DNA modifications leads, via more frequent excision, to chromosomal fragmentation. On the other hand, we cannot exclude the possibility that there are conditions for increased 8-oxo-G incorporation into DNA in *mutT* mutants that would cause MutM/Fpg-dependent chromosomal fragmentation.

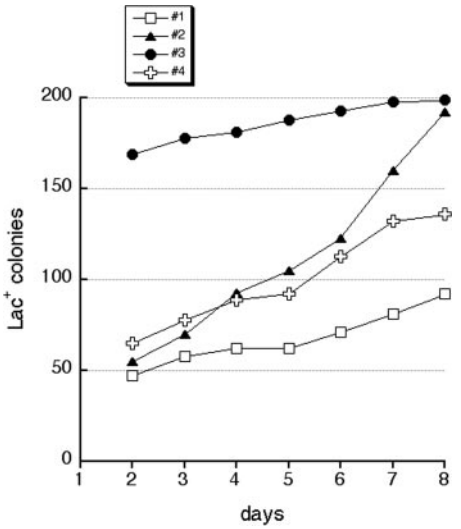

FIG. 9. Adaptive mutagenesis in CC101 strain. Four independent 12-ml cultures were grown in LB medium from fresh single colonies to saturation and plated on M9 plates supplemented with Lac and incubated for 8 days at 37°C. Each day, the total number of Lac^+ colonies on these plates was counted, and the results are shown in this graph. Culture 3 had an initial jackpot but then showed a similar rate of appearance of additional Lac^+ colonies as cultures 1 and 4, while culture 2 originally had a normal, low level of Lac^+ colonies but then showed an increased adaptive mutation rate relative to the other three cultures.

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