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The Antibiotic Trimethoprim Displays Strong Mutagenic Synergy with 2-Aminopurine

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ABSTRACT We show that trimethoprim (TMP), an antibiotic in current use, displays a strong synergistic effect on mutagenesis in *Escherichia coli* when paired with the base analog 2-aminopurine (2AP), resulting in a 35-fold increase in mutation frequencies in the *rpoB*-Rif^r system. Combination therapies are often employed both as antibiotic treatments and in cancer chemotherapy. However, mutagenic effects of these combinations are rarely examined. An analysis of the mutational spectra of TMP, 2AP, and their combination indicates that together they trigger a response via an alteration in deoxynucleoside triphosphate (dNTP) ratios that neither compound alone can trigger. A similar, although less strong, response is seen with the frameshift mutagen ICR191 and 2AP. These results underscore the need for testing the effects on mutagenesis of combinations of antibiotics and chemotherapeutics.

KEYWORDS antibiotic, mutagenesis, synergy, trimethoprim

Do drug combinations used in therapy have unforeseen effects on mutagenesis? Mutagenesis, mutagens, and the nature of mutations are topics that have long intrigued investigators (1–4; also, see reviews in references 5–7). Yet, only a handful of studies have focused on possible synergies between mutagens (8–15). It is important to identify increased mutagenesis that results from combinations of antibiotics and/or chemotherapeutics to allow improved risk assessment in employing these combinations and to possibly uncover new pathways of mutagenesis. In a recent study, we looked for synergies between different base analog mutagens and found a very strong synergy between 2-aminopurine (2AP) and zebularine (ZEB), and a strong antagonistic effect of 2AP on 5-bromodeoxyuridine (5BrdU) (16). We attributed the interaction of base analogs to the resulting changes in the ratios of deoxynucleoside triphosphates (dNTPs) that cause increased mutagenesis, in some cases at specific sequences (16). There is extensive literature from studies of both microbial and higher cells showing that alterations of the dNTP pools can affect mutation rates (17–29). In the work reported here, we describe strong synergistic effects that result from the combination of trimethoprim (TMP), an antibiotic in current use, and 2AP. We also show synergistic effects with 2AP and the intercalating frameshift mutagen ICR191. We analyzed the mutational spectra resulting from these pairwise combinations by using the *rpoB*-Rif^r system (30), an excellent system for examining the base substitution distribution of mutagens or mutators. It offers 92 mutations to examine that are fairly evenly distributed among the 6 possible base substitutions (16, 30–32). This is a large enough set to offer a number of sequence contexts for each base substitution but a small enough set to allow one to determine repeated occurrences of mutations at the same site and, thus, define hotspots (favored sites) and in so doing provide in many cases a fingerprint of a specific process. For example, replication errors have a certain fingerprint in this

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TABLE 1 *rpoB* mutant frequencies in wt and *mutS* strains with various mutagens in LB medium[a]

Strain	Treatment	Concentration (μg/ml)	No. of replicates	Frequency, f (10-8)[b] in *rpoB*	Mutation rate, μ (10-8)[b]	Ratio to untreated wt
wt	None		17	6.8 (5.8–14.8)	1.5 (1.3–2.8)	1.0
	TMP	0.15	20	122 (80.2–216)	22 (16–36)	14.7
	TMP	0.2	13	159 (94.4–221)	33 (21–43)	22
	TMP	0.25	47	125 (110–161)	33 (30–40)	22
	2AP	250	10	811 (508–980)	98 (65–120)	65.3
	2AP	500	25	899 (703–1430)	110 (88–170)	73.3
	TMP + 2AP	0.15 + 500	15	25,000 (10,230–60,400)	2,700 (1,200–6,100)	1,800
	TMP + 2AP	0.2 + 500	17	11,000 (6,230–17,700)	1,400 (830–2,100)	933
	TMP + 2AP	0.25 + 250	12	32,400 (19,800–65,200)	4,600 (3,400–8,500)	3,070
	TMP + 2AP	0.25 + 500	26	31,900 (23,300–46,500)	5,200 (4,000–7,200)	3,470
mutS	None		12	909 (638–1,690)	100 (75–180)	66.7
	TMP	0.15	16	11,400 (5,130–17,100)	1,200 (580–1,700)	800
	TMP	0.2	31	12,500 (6,290–33,400)	1,400 (760–3,400)	933
	2AP	500	20	3,060 (2,380–4,470)	350 (280–490)	233
	TMP + 2AP	0.15 + 500	18	42,000 (23,000–141,000)	4,100 (2,400–12,000)	2,730
	TMP + 2AP	0.2 + 500	32	129,000 (73,700–170,000)	13,000 (8,000–17,000)	8,670

[a]wt, wild type.
[b]Values in parentheses are 95% confidence limits.

system (30, 31), as do mutations occurring as a result of greatly altered dNTP ratios (18, 19, 29). Oxidative damage to guanines also has fingerprints in *rpoB*, as shown by the hotspots among the G:C→T:A transversion sites that occur in a *mutY mutM* double mutant and the AT→G:C transversions that occur in a *mutT* strain resulting from oxidizing dGTP triphosphates (30). The Rif system does not record frameshifts or large deletions but does record some in-frame short deletions or insertions (33). The analysis of base substitutions in this study allows one to record large increases in frequency that occur in the synergistic combinations here and to attribute their cause to shifts in dNTP ratios because of the resulting unique fingerprint in *rpoB*.

RESULTS

Mutagenicity and synergistic effects of TMP. TMP results in an increase in mutagenesis in *Escherichia coli*, as exemplified by an increase in rifampin resistance (Rif^r) (34). We can see this in Table 1, using the strain CC107 and three different concentrations of TMP. The addition of thymidine to the medium abolishes the mutagenic effect and also greatly reduces growth inhibition by TMP (Fig. 1 and 2). The mutagenic effect of TMP is not dependent on the SOS-induced translesion polymerases, as the Rif^r frequency is similar in a strain lacking all three translesion polymerases (translesion synthesis [TLS] deficient; SF2018) to the frequency of the starting strain (ZK126) (Table 2; see Materials and Methods). Moreover, the mismatch repair system is clearly involved in limiting the mutagenic effect of TMP, as the TMP treatment of a mismatch repair (MMR)-deficient derivative of CC107 (CC107*mutS*) results in greater than a 90-fold increase in the Rif^r frequency compared with TMP-treated CC107 and even an 11-fold increased frequency compared with the sum of the frequencies seen in the CC107*mutS* strain in the absence of TMP and in the wild-type CC107 in the presence of TMP (Table 1). TMP exhibits strong synergistic effects with the base analog mutagen 2AP. Table 1 shows that the combination of TMP and 2AP yields as much as a 30-fold increase in mutation frequency and a 35-fold increase in mutation rate over the additive effects of each individual mutagen. Although the mutational frequency of 2AP is not greatly increased in an *mutS* strain, the mutational frequency of the combination is increased in the MMR-deficient strain (the lack of a large frequency increase of 2AP alone in an *mutS* strain might be due to less efficient recognition of base analog mispairing than for mispairing of the standard base pairs).

Mutational spectrum of TMP-induced mutations. We sequenced the mutations in *rpoB* that result in the Rif^r mutants induced by TMP. The *rpoB*-Rif^r system allows us to monitor 92 different potential base substitution mutations that are fairly evenly dis-

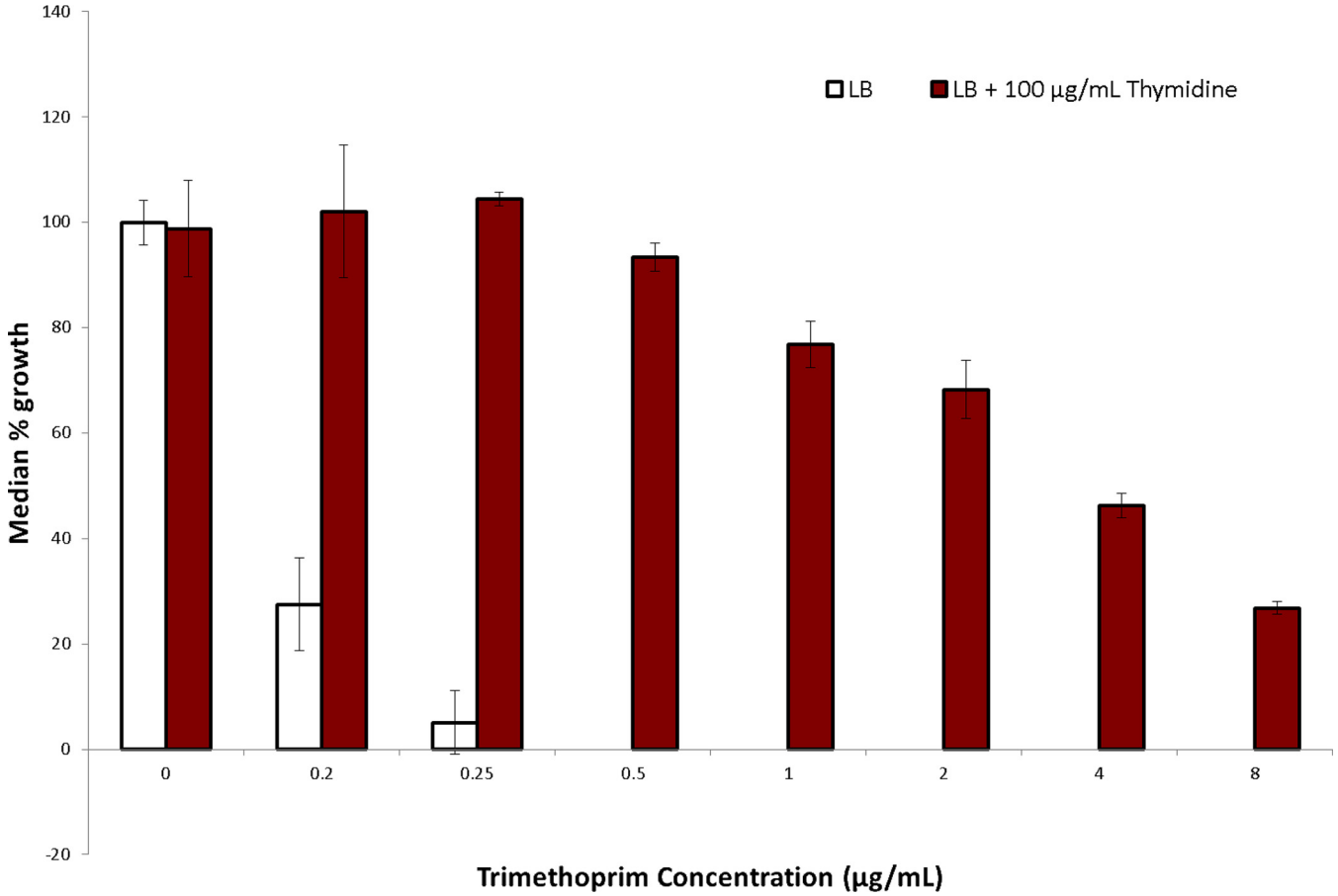

FIG 1 Growth potentiation by thymidine in the presence of different concentrations of TMP in LB medium. Error bars represent standard deviations.

tributed among the 6 possible base substitutions (16, 30–32). Figure 3 shows the spectrum of mutations in *rpoB* induced by TMP in a wild-type strain (upper portion of the panel). Several specific A:T→G:C transition mutations predominate (at nucleotide positions 1547 and 1534), but there are also two A:T→C:G transversion hotspots (nucleotide positions 1714 and 1715). This spectrum is strikingly similar to the spectrum of mutations in a deoxycytidine deaminase (DCD)-deficient strain that we reported previously (19) and which is reproduced in the lower portion of Fig. 3. The two A:T→C:G transversion hotspots occur in both spectra. This result similarity suggests that the thymidine deprivation occurring in a DCD-deficient strain and in a TMP-treated wild-type strain is responsible for the observed mutagenic specificity. The mutational changes can be generated by at least two different mechanisms, oxidative damage (35) or changes in the dNTP ratios. The two A:T→C:G transversions could result from the oxidized precursor 8-oxo-dGTP, whereas the A:T→G:C transition hotspots are the classic fingerprint of replication errors caused by alterations in dNTP ratios (18, 19, 29). When we examined the sequence changes of mutations induced by TMP in the MMR-deficient strain, the spectrum was similar but without the two transversion hotspots, at least in this smaller sample size (20 isolates; data not shown), and is in fact identical to that of a MMR-deficient strain in the absence of treatment (30, 31), even though TMP is increasing the frequency of mutations by 5- to 10-fold.

The spectrum of mutations occurring in the synergistic combination of TMP and 2AP is shown in Fig. 4. Here, the spectrum consists of both the two A:T→GC hotspots at 1547 and 1534 and a new A:T→G:C hot spot at 1598, the latter of which is not being prominently represented in the spectra of TMP or 2AP alone (Fig. 3 and 4). The main hot spot in the 2AP spectra, which we generated from 65 mutations sequenced in this

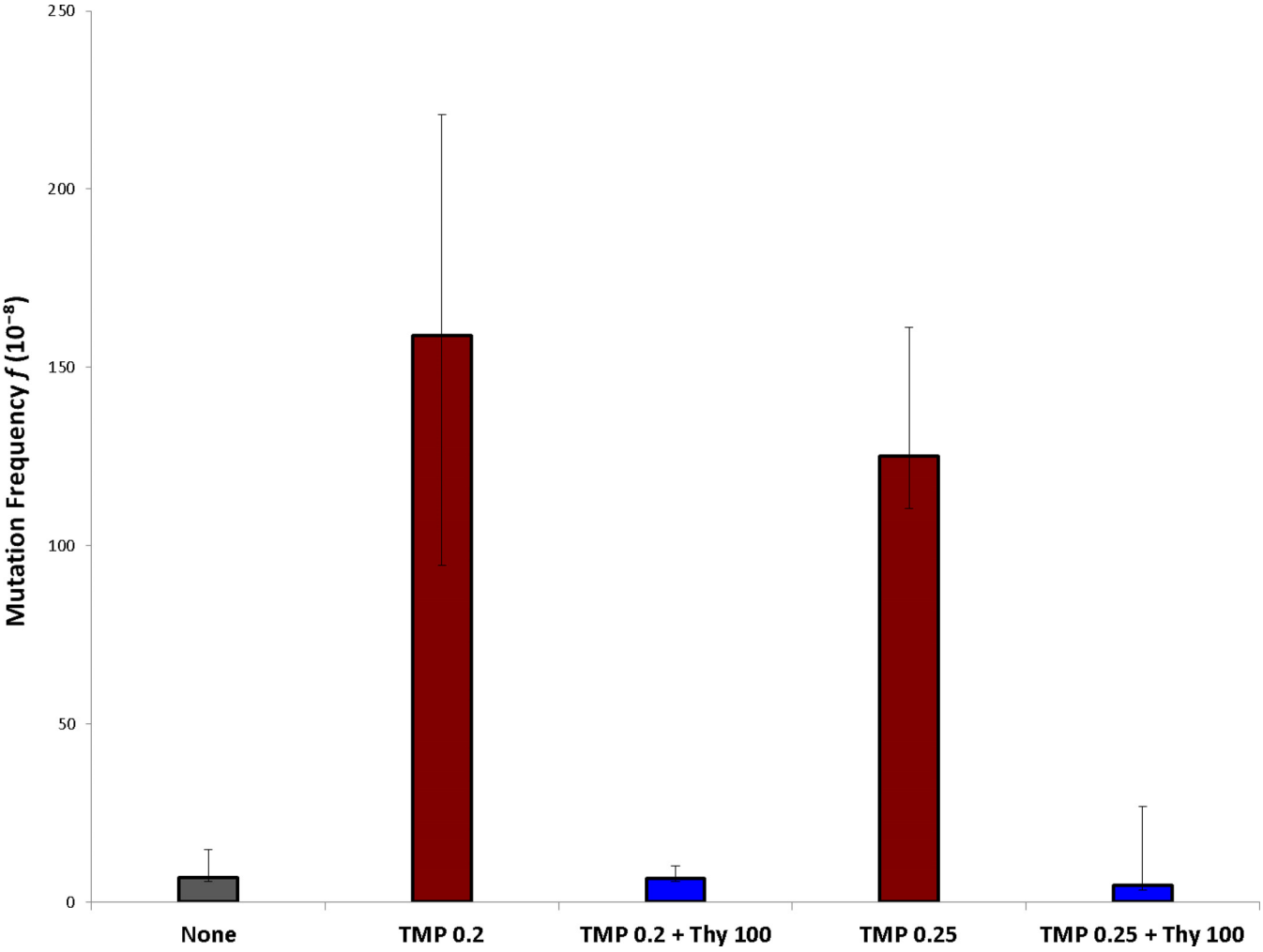

FIG 2 *rpoB* mutation frequencies in the CC107 strain background with TMP (0.2 to 8.0 μg/ml) in LB in the presence and absence of thymidine (100 μg/ml). Error bars represent 95% confidence intervals.

study plus 30 mutations from our previous study in the same background (30), is a G:C→A:T mutation at position 1576. When one considers the change in the ratio of the 1598:1576 peaks from the 2AP spectrum to the TMP + 2AP spectrum and the change in ratio of the 1598:1547 peaks from the TRM spectrum to the TMP + 2AP spectrum, as well as the 30-fold increase in the mutant frequency in the TMP + 2AP case, one can see the magnitude of the increase in the peak at 1598. This is the same situation we observed for the synergistic combination of the base analogs zebularine (ZEB) and 2AP (16; see Discussion). We note that the 30-fold increase in mutation frequency due to replication errors caused by dNTP ratio alterations would not be expected to include mutations caused by oxidative damage, which probably accounts for the failure to

TABLE 2 *rpoB* mutant frequencies in strains SF2018 and ZK126 with various mutagens in LB medium

Strain	Treatment	Concentration (μg/ml)	No. of replicates	Frequency, *f* (10-8)[a] in *rpoB*	Mutation rate, μ (10-8)[a]
SF 2018 (TLS deficient)	None		18	1.88 (0.6–3.5)	0.71 (0.32–1.1)
	TMP	0.1	17	22.3 (15.8–32.6)	5.0 (3.8–6.8)
ZK 126	None		18	3.40 (1.9–4.4)	0.90 (0.57–1.1)
	TMP	0.1	17	26.1 (3.9–41.8)	5.5 (1.2–8.2)

[a]Values in parenthesis are 95% confidence limits.

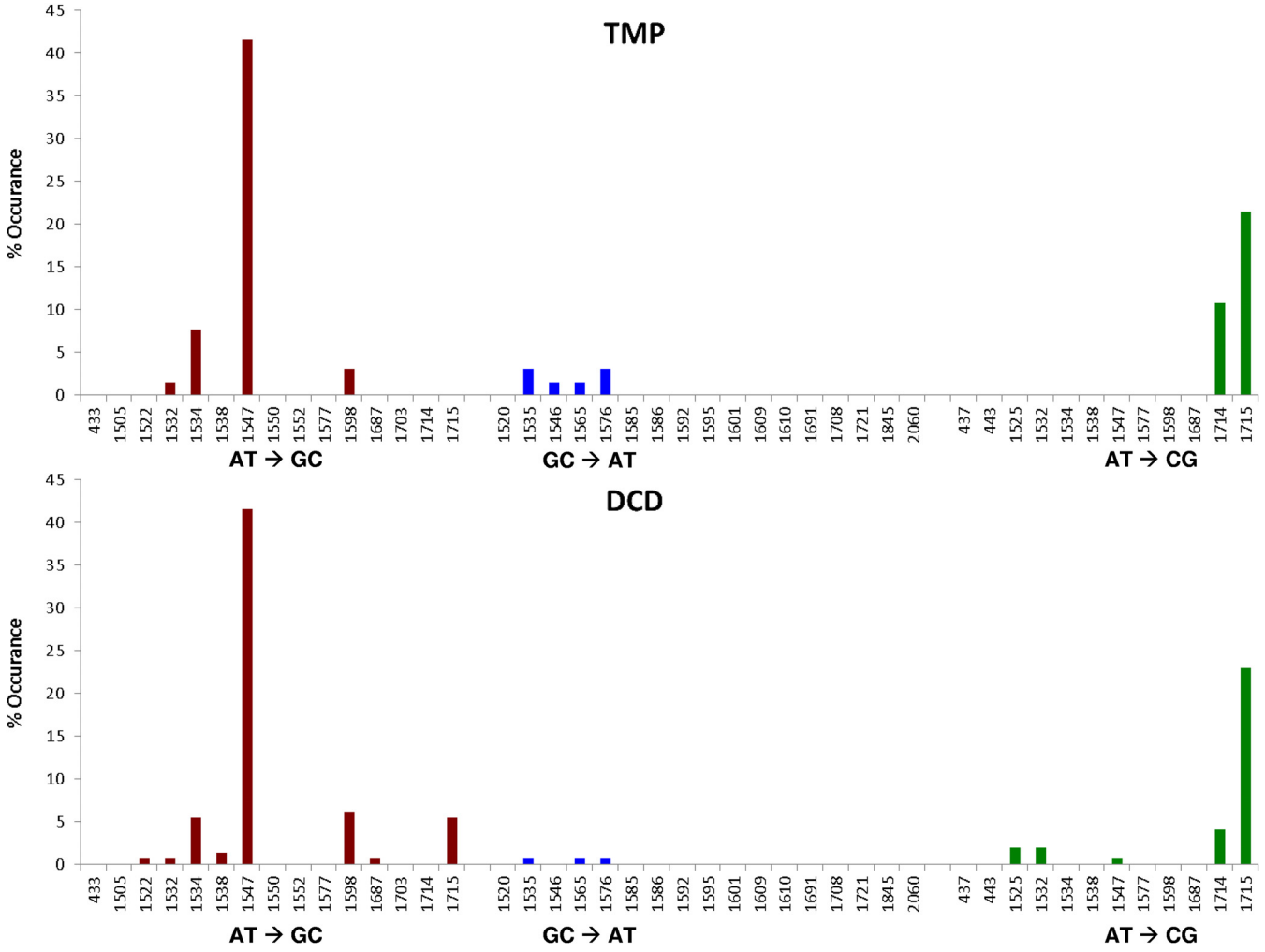

FIG 3 The spectrum of mutations in *rpoB* caused by TMP (top) and that occur spontaneously in a strain deficient for DCD (bottom; data from reference 19). The transitions (G:C→A:T, and A:T→G:C) are shown, together with the A:T→C:G transversions), as these represent 97% of all the mutations detected. The mutations are shown as a percentage of all the mutations detected in a particular sample (65 mutations for TMP and 146 mutations from the DCD-deficient background).

observe (Fig. 4) the two A:T→C:G transversions that are seen in the cells treated with TMP alone (Fig. 3).

Synergy with ICR191 and 2AP. We examined additional pairwise combinations of mutagens to look for possible synergistic or suppressive effects, using Rif^r to monitor mutation frequencies. There are technical challenges to this, as many combinations are strongly synergistic for killing and cannot be tested at concentrations that would be expected to promote mutagenesis. Thus, negative results cannot be easily interpreted. In any case, we failed to detect synergy between TMP and bleomycin and with either 4-nitroquinoline-1-oxide or cisplatin and any of the four base analogs, namely 2AP, zebularine, 5-azacytidine, and 5-bromodeoxyuridine. A significant synergy we did detect was with the combination of ICR191 and 2AP, which is detailed in Table 3. Although the level of mutations in the combination is not as high as seen for ZEB + 2AP (16) or for TMP + 2AP (Table 1), the 5- to 6-fold increase over that for 2AP alone is significant. Moreover, when we sequenced the mutations, we again detected the appearance of the three A:T→G:C hotspots at 1534, 1547, and, most importantly, 1598 (Fig. 5). The latter position (1598) appears to be a diagnostic signal for the type of synergistic process that we observe for all three strong synergies we have detected so far (ZEB + 2AP, TMP + 2AP, and ICR191 + 2AP; see Discussion).

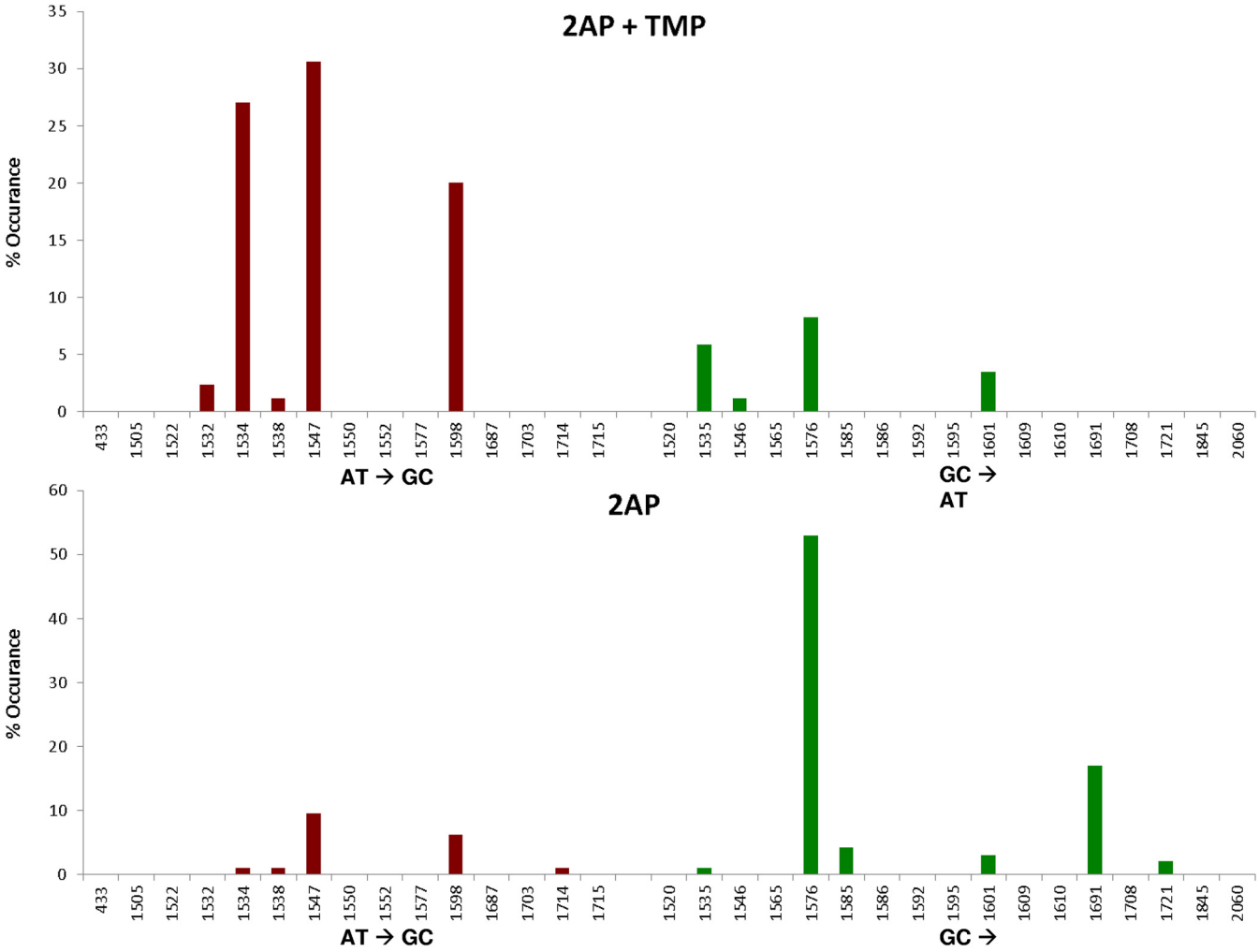

FIG 4 The spectrum of mutations in *rpoB* in strain CC107 caused by 2AP + TMP (top), and by 2AP alone (bottom; data from this work and reference 31). The transitions G:C→A:T and A:T→G:C are shown, as these represent 100% and 99%, respectively, of all the mutations detected. The mutations are shown as a percentage of all the mutations detected in a particular sample (85 mutations for TMP + 2AP and 95 mutations for 2AP alone).

DISCUSSION

The field of mutagen synergies is a vastly unexplored discipline, despite earlier work from several groups that pinpointed a number of specific synergies, in some cases involving compounds released into the environment (8–15). Some of these studies involved compounds that inhibit repair enzymes and, thus, act as mutagen enhancers (13, 15). Thus, in Chinese hamster lung fibroblasts, the mutagenicity of ionizing radiation and also of *N*-nitroso-*N*-methylurea is enhanced in the presence of formaldehyde, and this result is attributed to the inhibition of the enzymatic removal of the O^6-methylguanine lesions in DNA in the latter case, and to inhibition of excision repair in the former case (13). Similarly, sodium arsenite enhances the mutagenicity of UV radiation in TK6 human lymphoblastoid cells by impairing nucleotide excision repair (15). Other observed mutagenic synergies involve inhibiting enzymes that inactivate

TABLE 3 *rpoB* mutant frequencies in the wt strain with various mutagens in minimal medium with 1% LB

Treatment	Concentration(s) (μg/ml)	No. of replicates	Frequency, *f* (10-8)[a] in *rpoB*	Mutation rate, μ (10-8)[a]
ICR	10	29	7 (0–17)	2.4 (0–4.7)
2AP	250	23	1,300 (690–1,940)	190 (110–220)
ICR + 2AP	10 + 250	47	7,180 (6,160–7,950)	910 (790–990)

[a]Values in parenthesis are 95% confidence limits.

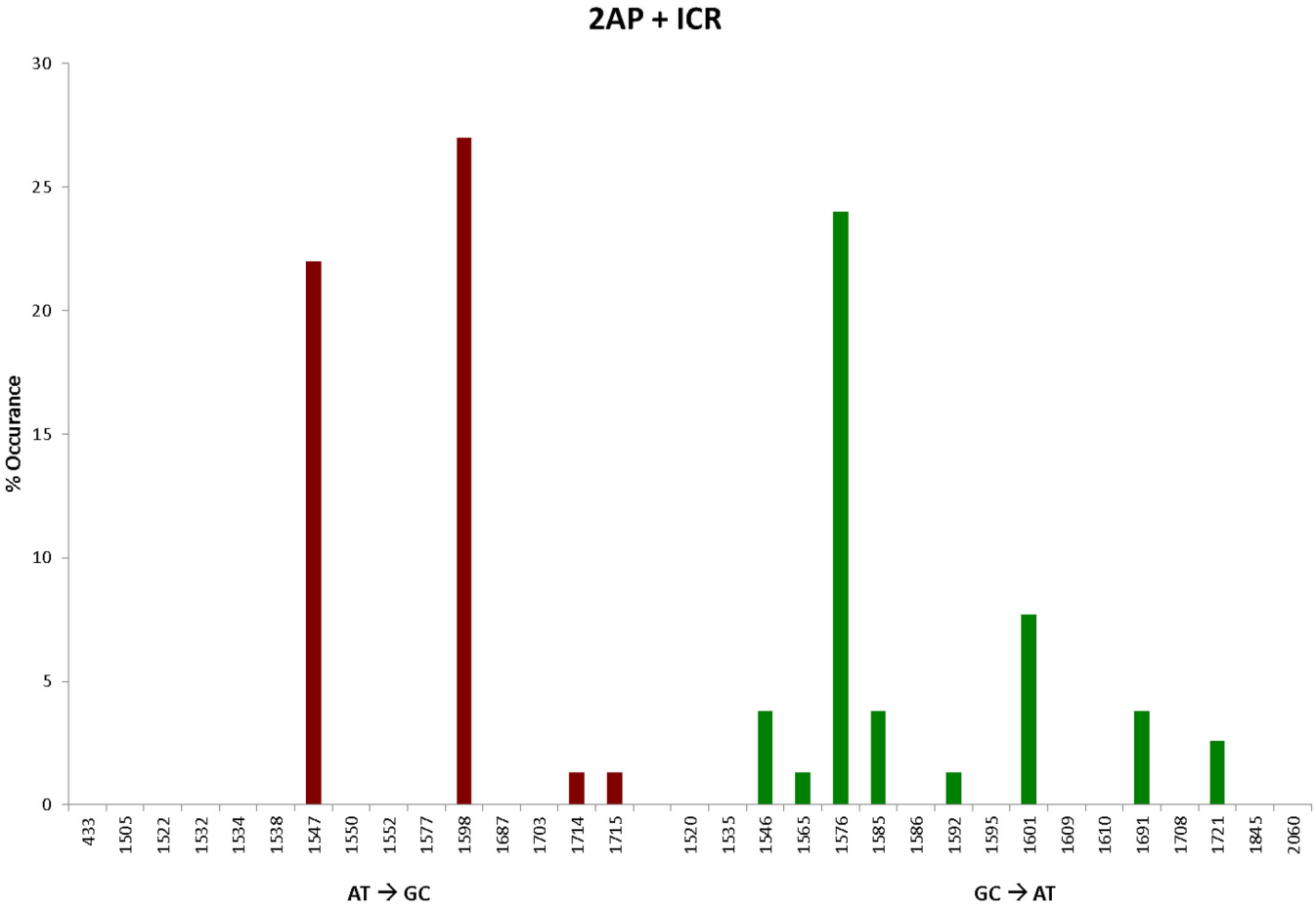

FIG 5 The spectrum of mutations in *rpoB* in strain CC107 caused by 2AP + ICR191. The transitions G:C→A:T and A:T→G:C are shown, as these represent 100% of all the mutations detected. The mutations are shown as a percentage of all the mutations detected in this sample of 77 mutations.

mutagens directly (10–12, 14). The pesticide pentachlorophenol (PCP) enhanced the mutagenicity of activated 2-aminofluorene (2AF) or of 2-acetoxyacetylaminofluorene (2AAAF) in a *Salmonella* tester system, presumably by reducing the rate of deacetylation degradation of the mutagen (10). Likewise, organophosphorus ester (OP) insecticides and their metabolites enhance the mutagenicity of heterocyclic amines (11) and a set of activated aromatic amines (12), although the exact mechanism is not clear but could involve effects on the stability of the mutagen or the repair of lesions. Black tea theafulvins also showed an enhancement of the mutagenic effect of aflatoxin B1 in a *Salmonella* tester system (14). The increase in mutagenicity has been attributed partly to conjugation with glutathione, resulting in decreased deactivation of the reactive intermediate aflatoxin B1 8,9-oxide (14). Finally, Hoffman and coworkers showed that intercalating agents enhance bleomycin mutagenicity in a yeast system and suggested that this may occur by increasing bleomycin accessibility to the DNA (8, 9).

We recently reported that certain base analogs displayed synergistic or suppressive effects when used in pairwise combinations (16). What differentiates this work from the studies described above is that the new synergy involves activating or initiating a novel process. In the work reported here, we have expanded on our initial findings (16) and try to elucidate some underlying concepts. The most striking effect in our first study involved the cytidine analog zebularine (ZEB) in combination with 2-aminopurine (2AP), which generated a 35-fold increase in mutagenesis compared with that of either compound alone. We argued that this effect results from changes in the dNTP ratios that result from these two compounds. Work from many laboratories over the past several decades has established that altering the dNTP ratios results in increased

replication errors that lead to increased mutations (17–29). An analysis of the spectrum of mutations induced in the *rpoB* gene by the ZEB + 2AP combination revealed the appearance of several hotspots for A:T→G:C mutations that are not prominent in the spectra of either mutagen acting alone (16). In particular, the appearance of a hot spot at position 1598 was noteworthy, as A:T→G:C mutations at this site are not well represented in the spectra of any mutagens or mutators resulting from gene inactivation in *E. coli* (19, 30, 31, 36). In the work reported here, we asked whether the synergy seen with ZEB + 2AP was rare and perhaps confined to pairs of base analogs or whether we could detect synergies involving other types of compounds, and in particular, compounds used in medicinal treatments. TMP is a widely used antibiotic that is an inhibitor of the *E. coli* dihydrofolate reductase that is responsible for the methyl group required for thymidine biosynthesis (37). TMP kills cells by starving for thymidine, resulting in thymineless death, a widely studied phenomenon (see references 38 and 39 and references therein; see also Fig. 1). TMP has been reported to have a low to moderate mutagenic effect in *E. coli* (34), although the mutations generated by TMP have not been extensively characterized. Here, we show that the mutational spectrum of TMP-induced mutations is identical to that of a mutant defective in deoxycytidine deaminase (DCD) (Fig. 2), an enzyme crucial to the synthesis of most of the thymidine in *E. coli*. (40–42). DCD-deficient strains grow slowly but are partially starved for thymidine. Most importantly, we find that the combination of TMP + 2AP gives a very strong synergy, generating a high level of mutations in *rpoB*, similar to that seen with ZEB + 2AP. Moreover, an analysis of the spectrum in *rpoB* reveals a profile similar to that seen for ZEB + 2AP, namely the three A:T→GC hotspots at positions 1534, 1547, and 1598. Mutations at position 1598 are not well induced by any mutagen or mutator so far examined but are prominent in the *rpoB* spectrum of a mutant with an engineered change in one of the allosteric sites of ribonucleotide reductase (29) that Schaaper and coworkers showed to also result in altered dNTP ratios, including increased dCTP and dGTP and lowered dTTP and dATP (29). Mutations at position 1598, as well as those at 1534 and 1547, are among those A:T sites in *rpoB* where the next nucleotide incorporated, after misincorporating a G across from the T is either a G or a C. At these sites, the misincorporation and procession of replication are enhanced when the dGTP and dCTP pools are increased. Considering that ZEB is an inhibitor of the mammalian and *E. coli* thymidylate synthases (43) and the addition of thymidine protects against ZEB induced killing (16), we can equate the effects of reduced thymidine with each of these two compounds (TMP, ZEB) and see that when paired with 2AP, the result is the appearance of AT→GC hotspots and, in particular, the A:T→G:C change at 1598 that seems to be an indicator of reduced thymidine in the presence of 2AP. The finding of synergy between ICR191 and 2AP is surprising, as is the emergence, again, of a peak of prominent mutations at position 1598 (Fig. 5). How one can explain the effects of a frameshift mutagen on dNTP pools that seems to be required to induce A:T→G:C changes at position 1598 remains an intriguing question for future study.

Taken together, all of the results here strengthen the idea that combinations of compounds used in different therapies (antimicrobial and anticancer) have the potential to cause severe mutagenic effects that are not easily predicted by their solitary activities; thus, there should be a concerted and organized effort to systematically test these combinations.

MATERIALS AND METHODS

***E. coli* strains.** Bacterial strains are listed in Table 4. The starting strain is CC107 (44), designated as wild type, which we have used in multiple mutagenesis studies (30, 36). This strain background is *ara (gpt-lac)5 thi*/F′128 *lacIZ proA*$^{+}$*B*$^{+}$. CC107 also carries a frameshift mutation in *lacZ* on the F′ plasmid. We have not observed any differences in the mutagenesis seen in this strain or in other wild-type strains we have used (16; see below). CC107*mutS* carries a miniTn*10cat* in *mutS* (36; Miller et al., unpublished). The starting wild-type strain used in Table 1 ZK126 (45) is W3310 Δ *lacU169 tna-2*, and its derivative, SF2018 (46), is translesion synthesis (TLS) deficient, as it is deficient in all three SOS-induced DNA polymerases (Pol II, Pol IV, and Pol V), is ZK126 *polB*::Spc^{r} *dinB*::Kan^{r} *umuDC*::Cam^{r}. These two strains were a gift from Steven E. Finkel.

TABLE 4 Bacterial strains described in this report

Strain	Genotype	Key phenotype	Reference
CC107	*ara* Δ(*gpt-lac)5 thi/*F′128 *lacIZ proA*$^+$*B*$^+$ (carries a +1 frameshift in *I* portion of *I-Z* fusion)		44
CC107*mutS*	CC107*mutS*::miniTn*10cat*	MMR deficient	36
ZK126	W3310 Δ *lacU169 tna-2*		45
SF2018	ZK126 *polB*::Spc^r *dinB*::Kan^r *umuDC*::Cam^r	TLS deficient	46

Media. The following media (47) were used. All the experiments with trimethoprim were carried out in LB (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter). Experiments with ICR191 were carried out in minimal A medium [10.5 g K2HPO4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate·$2H_2O$ per liter] supplemented with 10 ml of 20% glucose, 1 ml of 1 M $MgSO_4$, and 0.5 ml of 1% thiamine hydrochloride (vitamin B_1), and 1% LB per liter.

Growth conditions. Unless otherwise stated, all genetic methods were conducted as described by Miller (47; see also reference 48). Overnight cultures containing different concentrations of a given mutagen were seeded with approximately 500 to 1,000 cells by inoculating 2-ml cultures with 50 μl of a 10^{-4} dilution of a growing culture. After a 18-h incubation at 37°C on a rotor at 50 rpm (0.44 g), the cells were plated onto specific medium.

Determination of mutant frequencies. The cells grown as indicated above were plated onto LB plates with or without 100 μg/ml rifampin. The frequencies of Rif^r mutants were determined as described previously (30, 36). Briefly, mutant frequency (*f*) was determined as the median frequency from a set of cultures (the number of cultures varied from 12 to 65), and the frequency was also used to calculate the mutation rate (μ) per replication by the method of Drake (49), using the formula $\mu = f/\ln N\mu$, where *N* is the number of cells in the culture. The 95% confidence limits were determined according to the method of Dixon and Massey (50).

Chromosomal DNA isolation and sequencing. Chromosomal DNA was isolated from overnight cultures of each mutant after single-colony purification by streaking. The PCR tests were carried out as colony PCRs. The *rpoB* gene was PCR amplified from genomic DNA, using *Taq* polymerase (Bio-Rad) and sets of primers which allowed us to sequence directly from the PCR product. The sequences of the primers used for amplifying the *rpoB* encoding gene were as follows for the main set of mutations (cluster II): 5′-CGTCGTATCCGTTCCGTTGG-3′ and 5′-TTCA- CCCGGATAACATCTCGTC-3′. The unpurified PCR product was outsourced to Laragen (Culver City, CA) for purification with ExoSap (Affymetrix, USA) and sequencing. The following primer for the main cluster II was used for sequencing: 5′-CGTGTAGAG CGTGCGGTGAAA-3′. All of the mutations resulting in Rif^r mutants were detected with this primer. Mutant sequences were compared to the *rpoB* wild-type sequence using ApE (M. Wayne Davis) software.

Chemicals. Rifampin (Rif), 2-aminopurine (2AP), and trimethoprim (TMP) were purchased from Sigma (St. Louis, MO). ICR191 was purchased from Polysciences, Inc. (Warrington, PA).

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