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## Extreme dNTP Pool Changes and Hypermutability in *dcd ndk* Strains

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### SUMMARY

Cells lacking deoxycytidine deaminase (DCD) have been shown to have imbalances in the normal dNTP pools that lead to multiple phenotypes, including increased mutagenesis, increased sensitivity to oxidizing agents, and to a number of antibiotics. In particular, there is an increased dCTP pool, often accompanied by a decreased dTTP pool. In the work presented here, we show that double mutants of *E. coli* lacking both DCD and NDK (nucleoside diphosphate kinase) have even more extreme imbalances of dNTPs than mutants lacking only one or the other of these enzymes. In particular, the dCTP pool rises to very high levels, exceeding even the cellular ATP level by several-fold. This increased level of dCTP, coupled with more modest changes in other dNTPs, results in exceptionally high mutation levels. The high mutation levels are attenuated by the addition of thymidine. The results corroborate the critical importance of controlling DNA precursor levels for promoting genome stability. We also show that the addition of certain exogenous nucleosides can influence replication errors in DCD-proficient strains that are deficient in mismatch repair.

### INTRODUCTION

The size and balance of the deoxynucleotide triphosphate (dNTP) pools are important for replication fidelity (see review by Kunz *et al.* 1). Not only do unbalanced pools provoke an increase in mutagenesis (1–9), but an increase in the total pools of all four dNTPs also leads to increased mutations (10,11), while a decrease leads to reduced mutagenesis (12,13). DCD (deoxycytidine deaminase) carries out the first step, a deamination, in converting phosphorylated derivatives of deoxycytidine ultimately to deoxythymidine (14–18; see Figure 1). In *E. coli*, and in other gamma proteobacteria, this step occurs at the triphosphate level (*e.g.* 17,18), deaminating dCTP to dUTP, which is then hydrolyzed to dUMP. In most Gram positive bacteria, and in yeast and higher cells, this step occurs at the monophosphate

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level, deaminating dCMP to dUMP (19,20; see 2). The direct effect of deleting the *dcd* gene is the buildup of dCTP pools and, in some cases, the lowering of dTTP pools (2–4,18,21–22). As a result, this leads to increased mutagenesis in cell culture (5), yeast (2,21), and *E. coli* (3), to reduced growth rates (*e.g.* 23), and in yeast to moderately increased sensitivity to DNA damaging agents (2). Recently, we showed that DCD-deficient mutants of *E. coli* are hypersensitive to killing by exogenous cytidine, adenosine, or guanosine, and that this hypersensitivity is reversed by exogenous thymidine (24). We also reported that DCD-deficient mutants of *E. coli* are more sensitive to a series of different antibiotics, including vancomycin (25).

In the work described here, we show that double mutants lacking both DCD and NDK (nucleoside diphosphate kinase) have even more extreme imbalances of dNTPs than mutants lacking only one or the other of these enzymes. NDK is an important enzyme that is responsible for the conversion of the cellular dNDPs to the corresponding dNTPs. Despite its importance, loss of NDK ( $\Delta ndk$  strain) yielded a viable strain, indicating that it is not essential. It was shown that this is due the compensatory action of adenylate kinase (26). However, this compensatory effect leads to disturbed dNTP pools and a distinct mutator phenotype (3,6,9). Our present data reveal that the double *dcd ndk* mutant displays a particularly large increase in the level of dCTP and that this is accompanied by an exceptionally high mutation rate. Moreover, we show that the addition of exogenous thymidine or cytidine can influence replication errors.

## MATERIALS AND METHODS

### *E. coli* strains

The DCD-deficient strain used here is from the Keio collection, described in Baba *et al.* (27), made from the starting strain BW25113 (28). This starting strain (*lacI<sup>q</sup> rrnB<sub>T14</sub> ΔlacZ<sub>WJ16</sub> hsdR514 ΔaraBAD<sub>AH33</sub> ΔrhaBAD<sub>LD78</sub>*) is used as the wild-type (WT) in the experiments reported here, unless otherwise stated. The *dcd* mutant carries a complete deletion of the *dcd* gene, with a *kan* insert in place of the gene. The single mutants used in the experiments shown in Fig. 2 are also from the KEIO collection. Double and triple mutants were constructed by P1 transduction (28) using *ndk::mini-Tn10* or *mutS::mini-Tn10cam* (see also 9).

### Media

The following media (28) were used. LB (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter), minimal (minimal A; 10.5 g K<sub>2</sub>HPO<sub>4</sub>, 4.5 g KH<sub>2</sub>PO<sub>4</sub>, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g sodium citrate·2H<sub>2</sub>O). Minimal A is the buffer used for dilutions. For growth media, minimal A is supplemented with 10 ml of 20% glucose, 1 ml of 1 M MgSO<sub>4</sub>, and 0.5 ml of 1% thiamine hydrochloride (vitamin B1), per liter. Tryptone broth contains 10 g tryptone (1%) and 8 g NaCl per liter (24). The minimal medium A used here, when supplemented with tryptone, contains 1 g tryptone per liter (0.1%).

## ***E. coli* genetic methods**

Unless otherwise stated, all genetic methods are as described by Miller (29). The *dcd* mutant was purified from single colonies from the KEIO collection copy. Experiments were started by inoculating a fresh single colony of the *dcd* mutant. For experiments in LB, both wild-type and the *dcd* mutant were grown from single colonies during the day as a seed culture in LB supplemented with 50 µg/ml thymidine to a density of  $2-3 \times 10^8$  cells/ml. Overnight cultures were then seeded with approximately  $10^3$  cells by inoculating 2 ml cultures with 50 µl of a  $10^{-4}$  dilution of the over-day culture. Cells were then grown for 18 hours at 37°C on a rotor at 50 rpm. For experiments in minimal medium, the over-day culture in LB was spun down, washed, and resuspended in minimal A buffer before diluting  $10^{-4}$  and seeding overnight cultures.

## **Determination of mutant frequencies**

We inoculated 100–1000 cells in a series of cultures of LB that were then grown for 18 hours at 37°C with aeration, prior to plating on the appropriate medium (LB plates with or without 100 µg/ml rifampicin). The frequencies of Rif<sup>r</sup> mutants were determined as described previously (9,29). Briefly, mutant frequency (*f*) was determined as the median frequency from a set of cultures, meaning that each value represents the median of multiple determinations, from 9 to over 30 in each case. The mutation rate (*µ*) was determined by the formula of Drake (30). 95% confidence limits were determined according to the method of Dixon and Massey (31)

## **dNTP pool measurements**

dNTPs were extracted using the procedure described by Diaz *et al.* (32) with some modifications. Overnight cultures of each of four strains (WT, *dcd*, *ndk*, and *dcd ndk*) were grown at 37 °C in LB medium. The next day, each culture was diluted 1:2,000 in fresh LB medium and grown at 37°C with shaking to OD<sub>600</sub> = 0.4. For each strain, one hundred ml culture volume was filtered through a 0.45 micron polycarbonate filter (Sterlitech). After washing with 10 ml of saline, the filter was transferred to a Petri dish containing 10 ml methanol. After 2 hrs at –20° C, the methanol extract was heated for 2 min in a 95 °C waterbath, followed by centrifugation for 15 min at 17,000 g. The methanol supernatant was removed via lyophilization, and the remaining residue containing the dNTPs was resuspended in 1 ml of water. This solution was extracted with 0.5 ml of chloroform, followed by re-lyophilization and a final resuspension in 0.1 ml H<sub>2</sub>O. Quantitation of extracted dNTPs was by reverse-phase ion-pairing chromatography, as described (33) with minor modifications. Typically, 30 µl of the sample was loaded on the column. Peaks for the individual dNTPs were identified based on retention times of dNTP standards as further confirmed by the recorded UV spectra for each peak. Quantitation was by peak area at 260 nm, corrected for the differential extinction coefficients of the various nucleotides at this wavelength. This experiment was performed three times on separate days, and the results for each strain were averaged.

## Chemicals

Kanamycin, tetracycline, chloramphenicol, rifampicin, vancomycin, nitrofurantoin, ciprofloxacin, cephradine, erythromycin, adenosine, guanosine, uridine, thymidine, and cytidine were purchased from Sigma (St. Louis, MO).

## RESULTS

### Mutator effect of DCD-deficiency in different strain backgrounds

DCD deficiency has previously been shown to cause a moderate mutator effect (2,3,5,21). Thus, in Table 1, one can see a 26-fold increase in the frequency of *rpoB* mutations leading to a Rif<sup>r</sup> phenotype, in line with previous findings (e.g. 3). The addition of exogenous thymidine (50 µg/ml) restored the mutation frequency to near wild-type levels. We have expanded the data set of DCD-deficient mutator effects by looking at *dcd* mutations in concert with cells lacking mismatch repair (*mutS*) or lacking nucleotide diphosphate kinase (*ndk*), which itself results in an imbalance of dNTPs (3,6). The *mutS* background has, in these experiments, an almost 200-fold higher mutation frequency than the wild-type (Table 1), and the double *dcd mutS* mutant has an additional 14-fold increase in Rif<sup>r</sup> frequency over the *mutS* mutant alone (Table 1). Strains lacking a functional *ndk* gene also show a moderate mutator effect (Table 1, and ref. 6,9), which is further enhanced by the *mutS* deficiency by about 10-fold (not shown here, but described in detail in ref. 9). Interestingly (see Table 1), the combination of *dcd* and *ndk* results in a synergistic effect, since the respective increases in frequency over wild-type of 26 and 38 for each single mutant now become 800-fold in the *dcd ndk* double mutant. This factor is modestly increased by 3.5-fold in the triple mutant *dcd ndk mutS*.

### Mutational spectra in the *rpoB* gene

We used the *rpoB*/Rif<sup>r</sup> system to monitor base substitution mutations. In this system, any one of 80 different base-substitution mutations can result in resistance to rifampicin (Rif<sup>r</sup>; 34,35). Figure 2 displays the results for 725 Rif<sup>r</sup> mutants from the following six derivatives of the starting strain (28, see Materials and Methods): *mutS*, *dcd*, *dcd mutS*, *dcd ndk*, *ndk*, and *dcd ndk mutS*. (For sample sizes, see Legend to figure 2). We have previously reported the *mutS* spectrum in the *rpoB* system (34,35), and analyzed *dcd* with the *lacZ* system (3). In *rpoB*, there are several prominent hotspots for replication errors that involve A·T→G·C transitions. In the *mutS* strain, the A·T→G·C transition at position 1547 is the most significant hotspot, with 1534 acting as a secondary hotspot (Figure 2, see also 34,35). *Dcd* strains also have the primary A·T→G·C hotspot at 1547, but in addition have a new hotspot for A·T→C·G transversions at position 1715. However, in the *dcd mutS* background, the increased mutagenesis (Table 1) results in a shift towards the same hotspots found in *mutS* alone. The *dcd ndk* background, which has greatly increased mutagenesis over *dcd* or *ndk* alone, shifts to a pattern with the 1547 A·T→G·C as the major hotspot, but also has prominent peaks among several A·T→T·A sites. Analysis of *ndk* strains has previously shown that A·T→T·A mutations are well represented (9), as are A·T→G·C mutations at the 1547 hotspot (9). The spectrum for the *dcd ndk mutS* strain mirrors that of *mutS* alone.

## Effect of Nucleosides on Replication Errors

The finding that the addition of thymidine greatly lowers the mutagenic effect of *dcd* strains (Table 1; see also work in cell culture, 5) led us to ask whether the addition of other nucleosides also have effects. We recently reported that addition of cytidine, adenosine, or guanosine kills *dcd* strains of *E. coli*, presumably due to extreme thymine starvation (24). Therefore, we tested what the effects of nucleosides would be on strains with an intact *dcd* gene. We first looked at strains lacking mismatch repair (*mutS*), and these results are shown in Table 2 using Rif<sup>r</sup> mutants as an indicator. Here, one can see that the addition of cytidine results in a significant, 2.5-fold, increase in mutations (Student' t-test  $p = 3.12\text{E-}07$ ), while the addition of thymidine results in a 2.5-fold decrease ( $p = 4.35\text{E-}14$ ). The additions of adenosine, guanosine, or uridine do not result in a significant change ( $p = 0.724, 0.282$ , and  $0.541$ , respectively). The effects of cytidine and thymidine are evident in *mutS* strains, but are not observed in *mutY* strains or in wild-type strains with an intact mismatch repair system (Table 3). When we looked at the effects on the mutational spectra in *rpoB* of adding thymidine, we found no detectable change in the spectra for either *dcd* or *mutS* strains (Figure 3), even though the frequencies are affected (Tables 1 and 2).

## Measurement of dNTP levels

To investigate whether the extreme mutability of the *dcd ndk* mutant is due to significant disturbances in the intracellular dNTP pools, we determined the dNTP levels in the respective strains. The results are shown in Table 4. The *dcd* and *ndk* single mutant strains are characterized by modest, but distinct changes in their dNTP pools that are generally consistent with a previous report on these mutants in a different strain background (3), although some differences are also noted. As before, the *dcd* mutant shows an elevated dCTP pool and a slightly reduced dTTP pool. The *ndk* mutant also displays an elevated dCTP pool, but its dTTP pool is increased. A reduction in dATP level, which was noted previously for the *ndk* strain (3), was not observed here. Importantly, the *dcd ndk* double mutant strain showed further dramatic changes in dNTP pools. In particular, there is very strong expansion of the dCTP pool (18-fold). At this level, dCTP is the highest of all nucleotides, even exceeding ATP by more than 2.5-fold. The double mutant also shows further elevations of the dGTP and dTTP pools, and a significant drop (5-fold) in the dATP level. It is likely that these substantial changes in the dNTPs are responsible for the very strong mutator effect observed for the *dcd ndk* double strain (see Discussion).

## DISCUSSION

*E. coli dcd* mutants grow slowly in the absence of thymidine because the primary pathway for thymidine synthesis involves the DCD-catalyzed deamination of dCTP, which is now blocked (see Figure 1). The second route, via RNR reduction of UDP→dUDP followed by the NDK-catalyzed conversion to dUTP normally provides only 20% of the normal amount of thymidine in *E. coli* (41, and references therein). Adding even very low levels of thymidine improves the growth of *dcd* strains to near normal levels. Direct measurements show that DCD-deficient *E. coli* cells have increased levels of dCTP (3,18) and decreased levels of dTTP (18), leading to increased mutation rates (3), as has also been found in yeast (21) and cultured mammalian cells (4,22,36). Here, we have examined mutants doubly-

deficient in both DCD and NDK. Lack of NDK in *E. coli* has also been shown to cause dNTP pool alterations and elevated mutability (3,6,9), although distinct from that of *dcd* (3). We found that the double-mutant strains display extremely elevated levels of dCTP, along with dramatically increased mutability compared to the single mutants (Tables 1 and 4). The study provides a striking example of how important it is for cells to control and contain their dNTP levels, which are normally kept low (in the micromolar range) relative to the much higher (millimolar) levels of the RNA precursors. The very low dNTP concentrations suffice for synthesis of only a very small fraction of the genome, but it is clear that they need to be kept at this low level to enable faithful DNA synthesis. In the following, we will aim to correlate the dNTP pool changes (in particular of dCTP) with the increase in mutagenesis and the specific mutational changes observed in the *rpoB* target.

### Correlation of mutation rates with dNTP pool alterations

Our measurements of the dNTP pools (Table 4) show a number of different changes in the dNTP levels and proportional composition, but most noticeable are the increases in dCTP: about 3- and 4-fold in the single *dcd* and *ndk* mutants, respectively, and 18-fold in the double *ndk dcd* mutant. This results in a very high dCTP level, which exceeds even the ATP level by several-fold. Other dNTPs are also subject to changes, but to a lesser extent and less consistently. dCTP's tendency to 'run away' is likely due to the lack of feedback inhibition on the Ribonucleotide Reductase (RNR), which performs the essential reduction step of NDPs to dNDPs, and which is a critical step in the regulation of the dNTPs. RNR is subject to feedback control by (d)ATP, dTTP, and dGTP, but not dCTP (37). The corresponding increase in the frequency of Rif<sup>r</sup> mutations is also very large. The frequencies in the range of  $5,000 \times 10^{-8}$  of Table 1 represent a 3,000-fold increase over the control and are within a few-fold from the highest frequencies reported in this system (9,34,38,39).

Analysis of the precise types of mutations induced by dNTP pool changes is potentially complex. First, in duplex DNA, potential events at either DNA strand need to be taken into account. For example, for A·T→G·C base substitutions, polymerase errors could occur at the adenine or the thymine base. Second, at every DNA base, one base is always correct and three are incorrect, and one needs to consider each of the three competitions. Finally, the DNA sequence context plays a critical role. In particular, the 'next-nucleotide effect' has been shown to be important: following a misinsertion error, removal of the erroneous base by the proofreading exonuclease can be prevented by rapid insertion of the next correct dNTP. Through this mechanism, elevated dNTP concentrations promote mismatch extension and can be strongly mutagenic (3,33,40).

While dNTPs other than dCTP are also subject to change, these are more modest relative to those for dCTP. In addition, a strong, positive correlation is observed between the dCTP level and the mutant frequency: for the *dcd*, *ndk*, and *dcd ndk* strains, respectively, the increases in dCTP concentration are 3-, 4- and 18-fold (Table 4), and these are associated with 26-, 38- and 800-fold increases in mutant frequency (Table 1). Thus, analysis of the mutational effects primarily in terms of the dCTP concentration seems appropriate.

In Table 5, we present calculated frequency increases for various subclasses of mutation in the *rpoB* gene, based on inspection of the *rpoB* spectra (Fig. 2). There are strong increases

for the group of A·T→G·C transitions in each of the *dcd*, *ndk*, and *dcd ndk* strains. A·T→T·A transversions are uniquely enhanced in strains carrying the *ndk* defect. A·T→C·G transversions are also increased for the single mutant strains, but not for the double mutant. The latter is presumably due to the very large increase in the prevalent A·T→G·C transitions (see below), such that no such mutants were found in the sequenced sample.

The largest group of mutations is comprised by the A·T→G·C transitions, representing 72%, 50%, and 75% of the total mutations in *dcd*, *ndk*, and *dcd ndk*, respectively, and this group is also subject to particularly strong enhancements: 68-, 62-, and 2,300-fold in the three strains, respectively. As can be seen in Fig. 2, this class of mutations contains one particularly strong hotspot at *rpoB* position 1547. This site was also noted to be a hotspot in the *rpoB* spectrum of a wild-type strain (34). It is likely that this site has features that facilitate polymerase errors, and its analysis may be informative. The increase in frequency for this particular site in the *dcd*, *ndk*, and *dcd ndk* strains is also large: 48-, 65-, and 3,100-fold, respectively. The local sequence (in the *rpoB* coding strand, as displayed in Fig. 2) is 5'-GTC-3' (mutated base underlined). This particular sequence is also present at the second, minor hotspot site, *rpoB* 1534. The underlying polymerase error is likely a T·G mispairing (template base underlined), which is generally the most frequent polymerase error (38). We then note that the next (correct) nucleotide to be incorporated at 5'-GTC-3' would be dCTP opposite the 5' G. This is illustrated in Figure 4, which shows the DNA sequence context around positions 1534 and 1547 and the T·G mispairing event, as well as the required insertion of dCTP in the next, one (1534) or two (1547), template positions.

Thus, the elevation of dCTP in our strain is expected to promote extension of the T·G mismatch (at the expense of its exonucleolytic removal). In the *dcd ndk* double mutant, there is the additional lowering of the dATP concentration (Table 5), which is the correct nucleotide at this site, and this drop in dATP will further promote the misinsertion of the dGTP, consistent with the very strong increase for this event in the double mutant strain. This analysis provides a satisfactory explanation how the increased dCTP concentration is found to be so strongly mutagenic. Alternatively, the mutagenic event could take place during replication of the noncoding strand, for which the local sequence would be 5'-GAC-3'. The mispairing error would be A·C and the next nucleotide to be incorporated would again be dCTP. Thus, both strands could contribute, although their relative contributions would depend on the relative importance of T·G vs. A·C mispairing.

A·T→T·A transversions are also enhanced by the pool alterations, particularly in strains containing the *ndk* deficiency (Table 5). The increase is 52-fold for the single *ndk* strain, and 935-fold for the *dcd ndk* double mutant. This increase for A·T→T·A transversions has been noted before (3,9), and likely results from increased T·T mispairings (3,38,41). These events are promoted by increased dTTP (incorrect) and decreased dATP (correct), and these particular pool changes are indeed observed, at least for dTTP in the *ndk* and *dcd ndk*, and for dATP in *ndk dcd* (Table 5).

The third class of enhanced *rpoB* mutations are the A·T→C·G transversions, 52- and 63-fold for *dcd* and *ndk* strains, respectively (Table 5). Interestingly, in the *dcd* strain this entails one strong hotspot (position 1715, where 32 events are scored out of 44 at the 12 sites at which

an A·T→C·G transversion can be scored), while in the *ndk* strain it involves another hotspot (position 1714, 7 out of 9 events). An A·T→C·G hotspot at 1714 was also observed in the wild-type (34,35). The adjacent A·T base pairs of positions 1714 and 1715 reside within the same 5'-GATC-3' sequence (although with opposite orientation; see 34). A genome-wide sequencing study of spontaneous mutations shows that 5'-GATC-3' sequences are hotspots for transversions at A·T base pairs (42). It is possible that these events are mediated by different mechanisms.

### Comparison with previous studies

Our study uses the *rpoB* system to the mutational specificity of the various mutator strains. The *rpoB* system has proven to be a very useful system for mutation analysis, allowing detection of all six base-pair substitutions via a total of 80 base-substitution pathways at over 40 different sequence sites throughout the *rpoB* gene (35). Nevertheless, like all mutational detection systems it has a built-in specificity resulting from its intrinsic DNA sequence, which is usually revealed by a pattern of mutational hot (and cold) spots. The *rpoB* position at 1547 is an example of such a hotspot. Previous studies on *dcd* and *ndk* mutants have additionally used *lacZ* reversion systems (3,9). These systems afford an in-depth view into individual base-substitution pathways, however in only one specific DNA sequence context, the GAG codon encoding Glu-461 in *lacZ* (43). For example, using this system, the *ndk* deficiency was shown to preferentially promote A·T→T·A transversions (3,9), while the A·T→G·C transition at this same codon was only moderately promoted by the *ndk* deficiency. These results are not inconsistent with our current data: A·T→T·A transversions are also quite strongly induced in our *ndk* spectrum (52-fold, see Table 6), while the A·T→G·C transition at *lacZ* codon 461 is likely a relative cold spot due to its specific sequence context (it has no stimulatory dCTP effect), explaining the modest effect of the *ndk* deficiency in that system. With regard to *dcd*, the *lacZ* system showed strong increases of both A·T→T·A and G·C→T·A transversions, and a modest (3-fold) effect on the A·T→G·C transition (3). The positive response for the two transversions in the *lacZ* context could be readily accounted for by the observed dNTP imbalances (high dCTP, low dGTP) in the given DNA sequence contexts (3). It is likely that the strong A·T→G·C hotspot at *rpoB* 1547 has limited the detectability of the two indicated transversions within the sample of sequenced *rpoB* mutations. It is also possible that small changes in the dNTP pools when comparing different strain sets (see Table 6) may influence the results. The data in Table 7 are important, as they highlight the likelihood that differences in nucleotide metabolism among different *E. coli* backgrounds may affect dNTP values. Finally, it is important to note that forward and reversion studies often have different goals: the reversion studies (3) were aimed at correlating dNTP pool changes with specific mutational responses, while the current *rpoB* study was aimed at understanding the dramatic mutability of the *dcd ndk* double mutant.

### The role of DNA mismatch repair

DNA mismatch repair, encoded by the *mthLS* genes in *E. coli*, is a powerful error-correcting system for DNA replication errors (44). However, one of its vulnerabilities is its limited capacity because the system can become overwhelmed (saturated) by increased levels of replication errors (45,46). It is likely that diminished effectiveness of the system is

a contributing factor in the mutant frequencies in the *dcd*, *ndk*, and, especially, the *dcd ndk* strain. While the effectiveness of mismatch repair in a wild-type background is near 200-fold (Table 1, compare wt to *mutS*), it is reduced to about 100-fold in the *dcd* background (Table 1, compare *dcd* to *dcd mutS*), while is only 3.5-fold in the *ndk dcd* strain (Table 1, compare *dcd ndk* to *dcd ndk mutS*). For the most prevalent mutations, the A·T→G·C transitions, the corresponding correction factors are 600 (wt), 140 (*dcd*), and 4.5 (*dcd ndk*). Thus, in the double mutant replication errors are prevalent enough to at least partially saturate the mismatch repair system and strongly curtail the system's effectiveness. Therefore, the very strong increases for some of the mutations in the *dcd ndk* strain, for example the 2,300-fold increase for the A·T→G·C transitions (Table 5), presumably overstate the actual mispair production by a significant measure. Hence, the correlations between dCTP increases and mutant frequency increases are likely to be more proportional than appears at first glance.

### The antimutagenic effect of thymidine

The data of Table 1 show that the presence of thymidine in the medium strongly reduces the *dcd* mutator effect. This result is consistent with the restorative effect of thymidine reported in many other studies of impaired thymidylate biosynthesis (reviewed in Kunz; 47). When nucleotide levels were measured, impairment of thymidylate synthesis generally showed strong increases in dCTP levels as well as reduced dTTP levels (47). Addition of thymidine, which permits dTTP production via the thymine salvage pathway, restored the dCTP/dTTP ratio and ameliorates many of the deleterious effects (2,21,24,47). Hence, many of these studies have been interpreted in terms of an altered dCTP to dTTP ratio.

In our case, we have not measured the dNTP pools upon addition of thymidine to the *dcd* strain. However, we note that our pool measurements (Table 4) show only a minor (~25%) diminishment of the dTTP pool for the *dcd* strain, while in the *dcd ndk* double mutant strain the dTTP level is actually increased (by about 2.5-fold). Overall, this is consistent with other measurements of dNTP pools in *dcd E. coli*, which have in several cases not shown large decreases in dTTP (3) (see also Table 6). In these studies, strains were grown in LB medium. As LB likely contains small amounts of thymine or thymidine, sufficient levels of such dTTP precursors may be available to keep dTTP at near normal level. In other cases where a decrease of dTTP is observed, such as in defined media, the increase in dCTP is quantitatively much larger than the decrease in dTTP (2,18,21). We therefore consider it unlikely that the lowering of dTTP pool is the major contributor to the mutator effects observed here. Instead, as argued above, mutability strongly correlates with the increase in dCTP.

Secondly, it follows that restoration of the dTTP pool is also not the likely explanation for the antimutagenic effect of thymidine for the *dcd* strain. Instead, we propose that addition of the thymidine negates the mutagenic build-up of dCTP. This possibility is supported by several previous studies in which a reciprocal, inverse relationship was demonstrated between dTTP and dCTP (5,49–53), including studies investigating the toxicity of high concentrations of added thymidine to cultured cells. In all cases, thymidine addition led to an expanded dTTP pool along with a severely depressed dCTP pool, inhibiting DNA

synthesis. The deleterious effects of thymidine could be circumvented by addition of deoxycytidine, which restores the dCTP level (50–52). Precisely how an increase in the dTTP pool causes depression of the dCTP pool is an interesting question. Plausible explanations involve effects of dTTP on Ribonucleotide Reductase (RNR). Specifically, dTTP can act as a negative (feed-back) regulator of RNR (37,54–55), and this is expected to reduce dCTP production. Second, dTTP may be a repressor of RNR transcription (49,53). The discovery of NrdR as a repressor for *E. coli nrdAB* (56) as well as the cell-cycle dependent regulation of *E. coli* RNR (57) are consistent with this possibility.

### The effects of added nucleosides

The result of Table 2 shows that the addition of nucleosides to wild-type cells can influence the mutation rates, at least in mismatch-repair-deficient (*mutS*) background, where the mutation rate more directly reflects the replication error rate. Specifically, cytidine increases the rate by 2.5-fold, while thymidine reduces it by about 2.5-fold. These results are consistent with the above-described effects of dCTP. Cytidine may be assumed to increase the dCTP concentration, which would be mutagenic, while thymidine, via its effect on dTTP, may lower dCTP, which would be antimutagenic. These effects are not seen in a wild-type strain or in a different mutator background (*mutY*, Table 3), consistent with the notion that in these backgrounds mutations originate from pathways other than uncorrected replication errors. It will be of interest to investigate these effects by direct measurements of the dNTP pools under these conditions.

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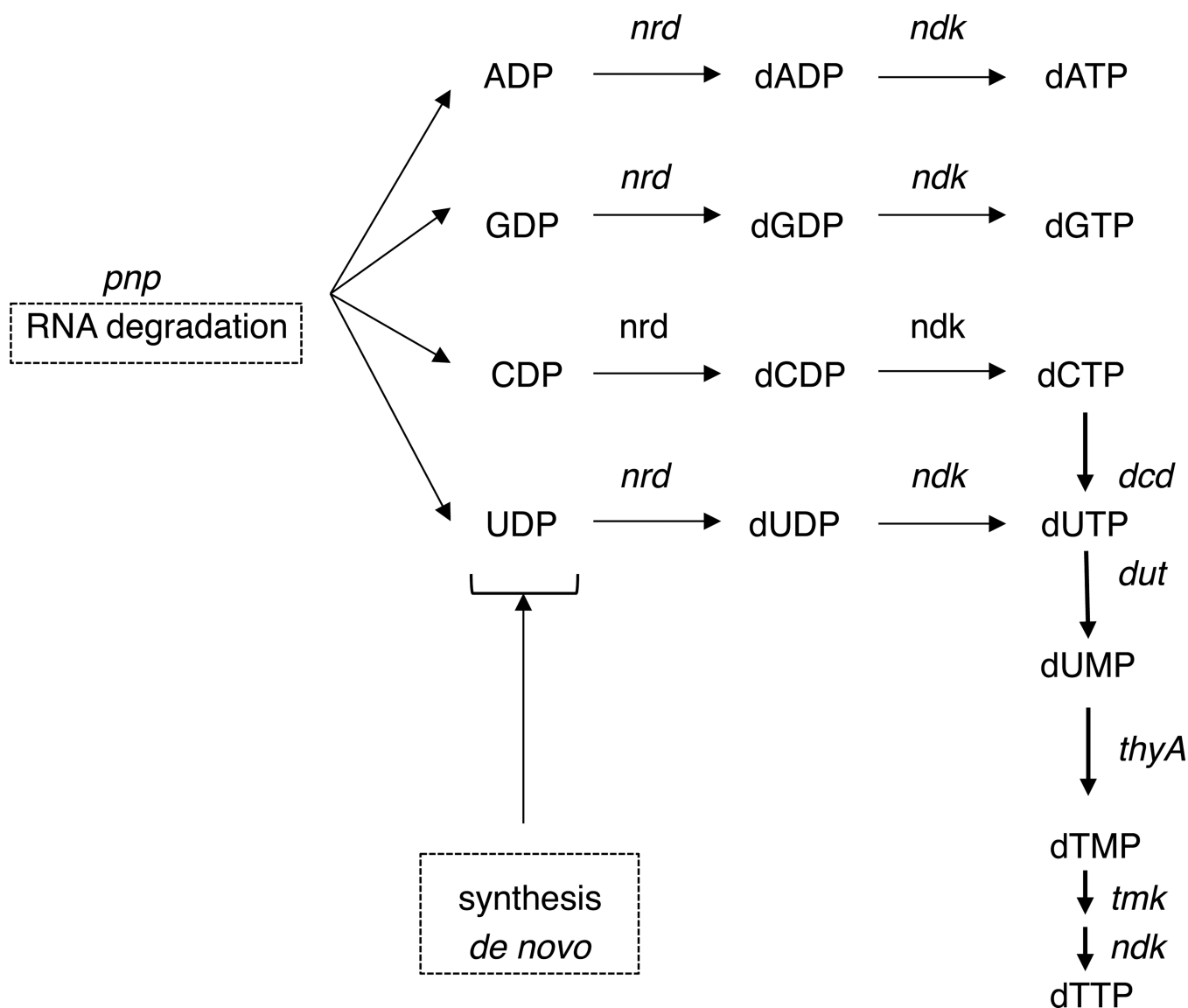
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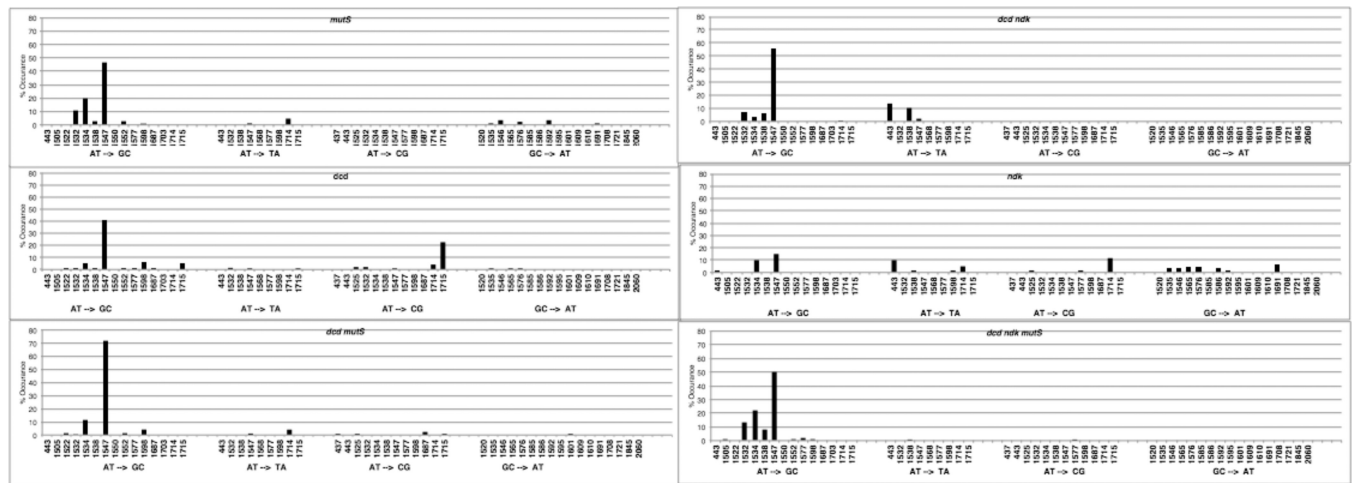
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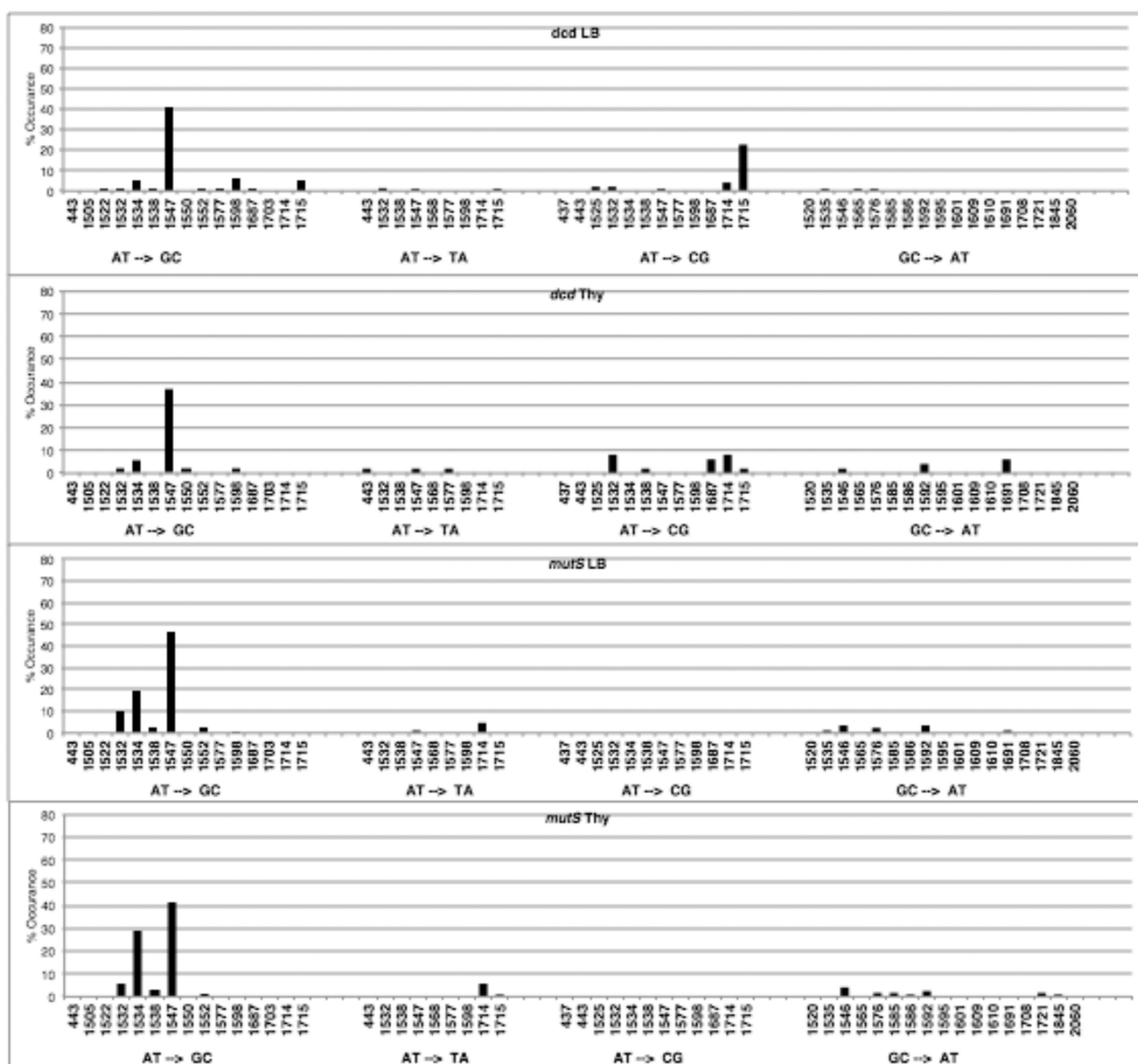


**Fig. 1.** Metabolic pathway for thymidine biosynthesis in *Escherichia coli*.



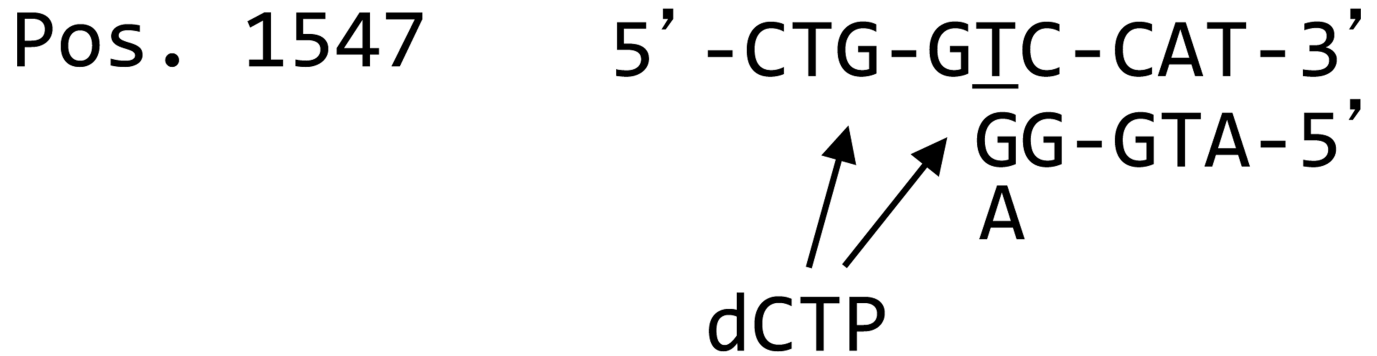
**Fig. 2.**

Distribution of mutations in *rpoB* for different mutant variations of the starting strain BW25113 (28). Mutations are shown at the *rpoB* sites as defined by Garibyan *et al.* (34). The number of occurrences is expressed as the percentage of all mutations analyzed for each sample. The sample sizes are: *mutS*, 88; *dcd*, 146; *dcd mutS*, 171; *dcd ndk*, 142; *ndk*, 61; and *dcd ndk mutS*, 117. Only four of the six base substitutions are represented here, as they represent virtually all of the mutations scored, including all 88 *mutS*, 145 of 146 *dcd*, all 171 *dcd mutS*, all 142 *dcd ndk*, 54 of 61 *ndk*, and all 117 *dcd ndk mutS*. Thus G:C → T:A and G:C → C:G transversions are not displayed.



**Fig. 3.**

Effect of exogenous thymidine on the mutational spectra. The effects of adding 100  $\mu\text{g/ml}$  thymidine to *mutS* and *dcd* strains is shown. The number of occurrences is expressed as the percentage of all mutations analyzed for each sample. The sample sizes are: *dcd*, 146; *dcd* + thymidine, 59; *mutS*, 88; *mutS* + thymidine, 126.



The next-nucleotide effect. Diagram showing how elevated dCTP levels affect the *rpoB* mutation rate at hotspot positions 1534 and 1547. In each case, the A·T→G·C transition responsible for the Rif<sup>r</sup> phenotype is represented as mediated by a T·G polymerase error. (The shown templates represent *rpoB* coding and non-coding strands, respectively, for 1534 and 1547). During the misinsertion event, the incorrect G is competing with the correct A. Following the misinsertion, competition ensues between the extension of the mismatch by incorporation of the next nucleotide dCTP, which would fix the mutation, with the exonucleolytic removal of the G. High levels of dCTP will push the forward reaction and, are therefore predicted to be mutagenic. Note that for the hotspot at position 1547 two dCTP incorporations follow the mismatch, consistent with the exceptionally high mutation rate for this site in response to elevated dCTP.

**Table 1**

The *rpoB* mutant frequencies (*f*) and mutation rate ( $\mu$ ) in various strain backgrounds<sup>b</sup>

| Strain              | Frequency ( <i>f</i> ) ( $\times 10^8$ ) <sup>a</sup> | Mutation rate ( $\mu$ ) ( $\times 10^8$ ) | Ratio to WT |
|---------------------|-------------------------------------------------------|-------------------------------------------|-------------|
| wt                  | 1.8 (1.3–2.4)                                         | 0.46 (0.36–0.58)                          | 1.0         |
| <i>dcd</i>          | 46.8 (39.4–62.8)                                      | 7.47 (6.44–9.63)                          | 26.0        |
| <i>dcd</i> (+ Thy)  | 3.1 (1.6–4.8)                                         | 0.693 (0.406–0.993)                       | 1.7         |
| <i>ndk</i>          | 68 (48–85)                                            | 10.1 (7.5–12.3)                           | 37.8        |
| <i>dcd ndk</i>      | 1,436 (1000–1810)                                     | 157.8 (114–194)                           | 798         |
| <i>mutS</i>         | 349 (322–374)                                         | 42.2 (39.3–44.9)                          | 194         |
| <i>dcd mutS</i>     | 5,070 (4500–6120)                                     | 509 (457–604)                             | 2,820       |
| <i>dcd ndk mutS</i> | 5,033 (4026–5942)                                     | 493 (402–573)                             | 2,790       |

<sup>a</sup>Values in parentheses are 95% confidence limits.

<sup>b</sup>All strains grown in LB except for one case of *dcd* mutants grown in LB supplemented with 50  $\mu$ g/ml thymidine (Thy).

**Table 2**

The *rpoB* mutant frequencies (*f*) and mutation rate ( $\mu$ ) of *mutS* strain in different media

| Medium                  | Frequency ( <i>f</i> ) ( $\times 10^8$ ) <sup>a</sup> | Mutation rate ( $\mu$ ) ( $\times 10^8$ ) | Ratio to LB |
|-------------------------|-------------------------------------------------------|-------------------------------------------|-------------|
| LB                      | 349 (322–374)                                         | 42.2 (39.3–44.9)                          | 1.0         |
| LB + thy 50 $\mu$ g/ml  | 145 (115–186)                                         | 19.3 (15.7–24.1)                          | 0.4         |
| LB + cyt 100 $\mu$ g/ml | 883 (802–960)                                         | 96.3 (88.3–103.9)                         | 2.5         |
| LB + ade 100 $\mu$ g/ml | 346 (320–450)                                         | 41.9 (39.1–53)                            | 1.0         |
| LB + gua 100 $\mu$ g/ml | 390 (328–1409)                                        | 46 (39.4–146)                             | 1.1         |
| LB + urd 100 $\mu$ g/ml | 405 (266–436)                                         | 47.9 (32.9–51.2)                          | 1.2         |

<sup>a</sup>Values in parentheses are 95% confidence limits. Abbreviations are thy (thymidine), cyt (cytidine), ade (adenosine), gua (guanosine), urd (uridine).

**Table 3**

The *rpoB* mutant frequencies (*f*) and mutation rate ( $\mu$ ) of wild-type and *mutY* in different media

| Medium                  | Strain      | Frequency ( <i>f</i> ) ( $\times 10^8$ ) <sup>a</sup> | Mutation rate ( $\mu$ ) ( $\times 10^8$ ) | Ratio to LB |
|-------------------------|-------------|-------------------------------------------------------|-------------------------------------------|-------------|
| LB                      | WT          | 1.8 (1.3–2.4)                                         | 0.46 (0.36–0.58)                          | 1.0         |
|                         | <i>mutY</i> | 29.3 (18.6–42.1)                                      | 4.78 (3.24–6.53)                          | 1.0         |
| LB + thy 50 $\mu$ g/ml  | WT          | 1.5 (1.1–2.1)                                         | 0.4 (0.31–0.52)                           | 0.8         |
|                         | <i>mutY</i> | 23.6 (22.1–27.9)                                      | 3.93 (3.71–4.54)                          | 0.8         |
| LB + cyt 100 $\mu$ g/ml | WT          | 1.8 (1.3–2.7)                                         | 0.44 (0.34–0.61)                          | 1.0         |
|                         | <i>mutY</i> | 28.5 (23.1–37.4)                                      | 4.54 (3.79–5.74)                          | 1.0         |

<sup>a</sup> Values in parentheses are 95% confidence limits.

Table 4

(d)NTP levels in *dcd* and *ndk* strains<sup>a</sup>

| Strain         | dCTP       | dGTP    | dTTP      | dATP    | ATP        |
|----------------|------------|---------|-----------|---------|------------|
| wt             | 145 ± 61   | 57 ± 37 | 163 ± 64  | 71 ± 13 | 919 ± 470  |
| <i>dcd</i>     | 348 ± 62   | 47 ± 37 | 120 ± 74  | 77 ± 19 | 1080 ± 370 |
| <i>ndk</i>     | 470 ± 143  | 95 ± 10 | 303 ± 71  | 81 ± 8  | 1225 ± 185 |
| <i>dcd ndk</i> | 2565 ± 211 | 81 ± 28 | 430 ± 133 | 14 ± 8  | 900 ± 196  |

<sup>a</sup>The numbers (± SD) are milliabsorbance units (A<sub>260</sub>) as measured by the HPLC instrument per OD<sub>600</sub> of the bacterial cultures at harvest, and represent the mean of three independent experiments.

**Table 5**

*rhoB* mutation frequencies ( $\times 10^{-8}$ ) for four individual classes of base-pair substitutions (in parentheses, fold increase over wt)<sup>a</sup>

| Strain              | Total       | AT→G·C      | AT→T·A    | AT→C·G    | G·C→A·T  |
|---------------------|-------------|-------------|-----------|-----------|----------|
| wt                  | 1.8         | 0.47        | 0.40      | 0.27      | 0.38     |
| <i>dcd</i>          | 47 (26)     | 32 (68)     | <         | 14 (52)   | <        |
| <i>ndk</i>          | 68 (38)     | 29 (62)     | 21 (52)   | 17 (63)   | <        |
| <i>dcd ndk</i>      | 1440 (800)  | 1080 (2300) | 275 (940) | <         | <        |
| <i>mutS</i>         | 350 (190)   | 280 (600)   | 20 (50)   | <         | 40 (125) |
| <i>dcd mutS</i>     | 5070 (2800) | 4500 (9500) | 207 (520) | 180 (660) | <        |
| <i>dcd ndk mutS</i> | 5030 (2800) | 4900 (4900) | <         | <         | <        |

<sup>a</sup>The frequencies for individual classes of mutations were calculated by multiplying the overall Rif<sup>r</sup> frequency by the proportion of mutants found for each class upon DNA sequencing (Fig. 2). < indicates that not enough events were scored (< 5) to calculate a reliable frequency or increase.

**Table 6**

Observed dNTP effects due to *dcd* deficiency in different *E. coli* strain backgrounds (wild-type level for each dNTP =1)

| Background          | dCTP | dGTP | dTTP | dATP |
|---------------------|------|------|------|------|
| Keio <sup>a</sup>   | 2.4  | 0.82 | 0.74 | 1.1  |
| KA796 <sup>b</sup>  | 3.7  | 0.5  | 1    | 1    |
| MC4100 <sup>c</sup> | 10   | 0.7  | 0.34 | 0.83 |

<sup>a</sup>Present study

<sup>b</sup>Schaaper and Mathews (3).

<sup>c</sup>Maslowska *et al.* (48).