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Determination of Cellular Pathways that Lead to Spontaneous and Induced Mutagenesis in *Escherichia coli*

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Determination of Cellular Pathways that Lead to Spontaneous and Induced Mutagenesis in *Escherichia coli*

A dissertation submitted in partial satisfaction of the

requirements of the degree Doctor of Philosophy

in Molecular Biology

by

Elinne Becket

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ABSTRACT OF THE DISSERTATION

Determination of Cellular Pathways that Lead to Spontaneous and Induced Mutagenesis in *Escherichia coli*

by

Elinne Becket

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2012

Professor Jeffrey H Miller, Chair

While much work has been done to elucidate the mechanisms through which mutations occur and the corresponding DNA repair, there is still much to be discovered in global pathways not directly related to the site of mutation, and how these pathways affect ensuing mutagenesis. In this dissertation, I will elucidate global pathways involved and how they influence both spontaneous and induced mutagenesis. We conducted a screen of six genotoxic agents against a single gene knockout library of *Escherichia coli*, and discovered a set of mutants that are involved in the cytotoxicity of these agents. In particular, folate biosynthetic pathways were found to be involved in the cell's resistance to a cytidine base analog. Next, using a papillation assay, we discovered four mutants involved in the nucleotide salvage pathway that show decreased levels of 5-azacytidine-induced mutagenesis, implicating RNA turnover and competing nucleotide pools in exacerbating this mutagenesis. We also show that the rNDP pool generated by the degradation of RNA is responsible for spontaneous mutations that result from replication errors, and these are normally repaired by the mismatch repair (MMR) system and

prevented by 8-oxo-dGTP diphosphatase. Our results suggest that the rNDP pools derived from RNA degradation fuel replication to the point that replication errors escape the exonucleolytic editing function of DNA polymerase, but can be dealt with by the MMR system. We propose that in the absence of the cell's primary exoribonuclease (polynucleotide phosphorylase), the reduced rNDP pools limit replication to the point where the editing function can correct replication errors, and the MMR system is not required. Additionally, we discovered that in the absence of this exoribonuclease, there is an increase in frameshift mutations in the mutational spectrum, possibly due to the minimization of normal replication errors and revealing secondary mutations.

The dissertation of Elinne Becket is approved.

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Chapter 2 – Frank Chen assisted in the screening of the KEIO collection against ICR-191. Cindy Tamae constructed the five double knockout strains used in this study.

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2. Liu A, Fong A, Becket E, Yuan J, Tamae C, Medrano L, Maiz M, Wahba C, Lee C, Lee K, Tran KP, Yang H, Hoffman RM, Salih A, and Miller JH (2011) "Selective Advantage of Resistant Strains at Trace Levels of Antibiotics: A Simple and Ultrasensitive Color Test for the Detection of Antibiotics and Genotoxic Agents." *Antimicr. Agents Chemother.* 55(3):1204-1210.
3. Becket E, Chen F, Tamae C, and Miller JH (2010) "Determination of Hypersensitivity to Genotoxic Agents Among *Escherichia coli* Single Gene Knockout Mutants." *DNA Repair*, 9(9):949-957.
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5. Tamae C, Liu A, Kim K, Sitz D, Hong J, Becket E, Bui A, Solaimani P, Tran KP, Yang H, and Miller JH (2008) "Determination of Antibiotic Hypersensitivity among 4,000 Single-Gene-Knockout Mutants of *Escherichia coli*." *Journal of Bacteriology* 190(17):5981-5988.

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CHAPTER 1 – A Review of DNA Damage and Repair

Mutations in DNA have been the subject of study since the elucidation of the genetic code, due to their implication in evolution and human disease ((1) and references therein, (2)). In this chapter I will review the factors contributing to induced and spontaneous mutations seen in DNA, as well as the DNA repair mechanisms that resolve these mutations.

Mutations – Driving Evolution and Disease

Mutations alter fitness, and the outcome of this fitness change is dependent upon the environment in which an organism lives (3). Since most mutations are deleterious (4, 5), their occurrence in organisms living in an unchanging environment leads to overall reduction in the fitness of the population. However, in a changing environment, mutations facilitate adaptation and are thus needed for the survival of the population (3, 6). It is for this reason that individual organisms have evolved a basal, balanced level of spontaneous mutations that allows for genetic variation in the event of selective pressure, but they keep this level low enough to minimize the incidence of fitness reduction resulting from deleterious mutations (Table 1-1 below, from (3)). This minimization is achieved through the evolution of complex and often redundant DNA repair pathways that correct damage and replication errors (7). However, this incidence of mutagenesis still results in deleterious mutations that lead to human disease (8-11). Point mutations within the three *ras* genes (GTPases involved in signal transduction) have been shown to convert them into active oncogenes, leading to a variety of cancers (12). Nussbaum and coworkers have linked Parkinson's disease with a base-substitution mutation within the human alpha-synuclein protein (13), and Rozen and coworkers have identified a similar mutation in 5,10-methylenetetrahydrofolate reductase, leading to hyperhomocysteinaemia and vascular disease (14). The instability of triplet repeat sequences

has also been implicated in human diseases like spinal and bulbar muscular atrophy, fragile X syndrome, and myotonic dystrophy (see Nucleotide repeat sequences later; (15)). Furthermore, Alu elements, which are transposable sequences that induce changes in the genome when re-inserted, have been linked to numerous human diseases such as hemophilia (16), breast cancer (17), and neurofibromatosis (18), through alteration of gene expression, disrupting reading frames, and splicing disruption (19). Additionally, the homology between these elements can lead to genome deletions between the two Alu sequences, which can lead to a variety of diseases if an important gene is within this deleted sequence (see Recombination Events for more detail; (19)).

Additionally, exogenous factors such as genotoxic agents and UV irradiation can also contribute to the increased incidence of these deleterious mutations, leading to a variety of human diseases including atherosclerosis and cancer (20-22). Given the increasing industrialization of modern society, these exogenous sources are of particular importance in the future of human cancer and disease.

Table 1-1. Relationship between species, genome size, and mutation rate (From (3)**)**

	Genome size (bp)	Mutation Rates[a] (mutations/basepair/replication)	(Mutations/genome/replication)
Riboviruses			
Bacteriophage Qβ	$\sim 3.5 \times 10^3$	1.9×10^{-3}	6.5
Poliovirus	$\sim 7.5 \times 10^3$	1.1×10^{-4}	0.8
Vesicular stomatitis	$\sim 1.1 \times 10^4$	3.2×10^{-4}	3.5
Influenza A	1.36×10^4	7.4×10^{-5}	~1.0
Retroviruses			
Murine leukemia virus	$\sim 8 \times 10^3$	3.3×10^{-5}	0.2
Human immunodeficiency virus type 1	9.75×10^3	2.1×10^{-5}	0.2
DNA-based			
Escherichia coli	4.6×10^6	5.4×10^{-10}	0.0025
Mus musculus	2.7×10^9	1.8×10^{-10}	0.49
Homo sapiens	3.2×10^9	5.0×10^{-11}	0.16

Mechanisms of DNA Repair

In order to minimize the frequency of mutations and avoid mutational decay, cells have developed numerous mechanisms to counteract and repair damage by mutagenic substances and processes. Here is an overview of these DNA repair processes (See Table 1-2 for summary (23)).

Detoxification – The Earth's atmosphere in early evolution contained very little O_2, and thus organisms first evolved to possess anaerobic metabolisms. As the atmosphere evolved to contain more oxygen, energetically, cells with strictly anaerobic metabolism cannot survive under oxygen-rich conditions cells. Thus cells evolved more efficient aerobic metabolisms that allowed them to take advantage of this energy source (24). However, with the presence O_2 comes toxic effects (25), as it is easily converted into reactive oxygen species, and with the use of aerobic metabolisms, cells have had to evolve proteins responsible for quenching these reactive compounds, namely superoxide dismutase (SOD) (26). Since reactive oxygen species are also involved in DNA damage and mutagenesis (described in detail later), these enzymes are key to preserving the integrity of the genome and eluding carcinogenesis. SOD enzymes contain metal ions that function to remove superoxide radicals from within the cell, converting 2 superoxide molecules into one hydrogen peroxide and one neutral O_2. Superoxide oxidizes 4Fe:4S clusters thus releasing iron which is kept reduced by reductants in the cell. This free iron then reduces hydrogen peroxide into the DNA-damaging hydroxyl radical, and thus *E. coli* SOD-deficient mutants that cannot quench superoxide are hypersensitive to hydrogen peroxide treatment (27, 28)(29). Additionally, cells have evolved catalases and peroxidases that eliminate hydrogen peroxide from the cell (24, 30).

Direct removal of lesions – Alkylation (31, 32) and the generation of photoproducts (23) from UV-irradiation are mutagenic lesions in DNA. The cell has evolved specific mechanisms for each lesion to split dimer photoproducts and remove unwanted methylation and bulky alkyl groups. The UV photoproducts are monomerized by photolyase enzymes, which are found in both single- and multi-cellular organisms, with the exception of humans and other mammals (33). This reduced enzyme binds directly to the DNA lesion, and using the energy from near-UV or blue light and chromophores such as a flavin protein and tetrahydrofolate (THF) for catalysis (34, 35), donates electrons to the dimerized pyrimidines, splitting them (33, 36).

Cells utilize additional enzymes to remove alkyl groups from DNA bases. An example is O^6-alkylguanine-DNA alkyltransferase, which removes alkyl groups from the O^6 position of guanine in a single step (37). Additionally, *E. coli* AlkB and respective human hABH2/3 remove alkyl groups from the N1 position of purines and the N3 position of pyrimidines (38, 39). Interestingly, AlkB and hABH3 also repair alkylated bases in RNA (38).

General excision repair – The nucleotide excision repair complex (UvrABC in prokaryotes, *XPA-G* in humans) is used for the general excision repair of DNA lesions (40). This complex uses separate functions to recognize and repair a wide variety of lesions including photoproducts, cross-links, bulky adducts, and abasic sites (41). One or more protein subunits constantly scan the genome, recognizing and binding to a damaged DNA region, and this binding then promotes the excision subunits to cleave DNA upstream and downstream of the damaged region (42, 43). This repair patch is then removed by a helicase and filled in with the correct sequence by DNA polymerase I (prokaryotes)/δ or ε (eukaryotes) and DNA ligase (42, 44). There are two types of nucleotide excision repair in eukaryotes: global excision repair, as described earlier and as seen in prokaryotes, and transcription coupled repair, which is enacted upon the transcription complex stalling when reaching regions of damaged DNA, which serves

as a damage recognition signal, the excision of the repair patch then proceeds as in global repair (40, 42).

Specific excision repair – While the nucleotide excision repair pathway is for the general removal of lesions, the base excision repair pathway begins through the activities of multiple DNA glycosylases that are each responsible for removing a specific subset of damaged DNA bases (45). These DNA glycosylases flip out damaged bases from the DNA and cleave the N-glycosidic bond, leaving an abasic site containing an intact deoxyribosephosphate backbone (although some bifunctional glycosylases also cleave the phosphodiester backbone) (45, 46). Glycosylase activities include the removal of uracil, alkylated-bases, and oxidized purines/pyrimidines, as well as the resolution of G/T(U) mismatches, adenine mismatches, and pyrimidine dimers (45). Furthermore, this activity does not cause a large local helical distortion as in nucleotide excision repair. Once these based have been removed the AP sites must be further processed, as they are mutagenic if unrepaired (47). In cases where the glycosylases do not cleave the phosphodiester backbone, an AP endonuclease performs this activity, leaving a single stranded break (45). Then a deoxyribophosphodiesterase removes the deoxyribosephosphate, leaving a single nucleotide gap, and this gap is repaired by DNA polymerase and ligase through either short- or long-patch repair (48-51)

Additionally, the GO system is evolved specifically to prevent and remove lesions created by oxidized guanine in DNA (8-oxo-dG). MutT in *E. coli* removes 8-oxo-(d)GTP from nucleotide pools (reducing them to 8-oxo-(d)GMP, which cannot be elevated to the diphosphate form), preventing the misincorporation of this oxidized base in DNA and RNA (52). MutY and MutM are glycosylases that repair the 8-oxo-dG lesions already present in DNA. MutY removes the mispaired adenine from a G:A mispair, allowing for the insertion of the correct cytosine residue (53), and MutM subsequently removes the oxidized base from the lesion via the base excision repair pathway (54, 55).

Post-replication – Once replication of the genome has commenced, DNA repair processes work to repair lesions that permit the completion of replication, as well as repair errors resulting from replication.

The mismatch repair system is comprised of two mechanisms, long patch mismatch repair (MMR, methyl-directed in *E. coli*) (56, 57) and very-short patch repair (VSP) (58, 59). VSP evolved to counteract the common deamination of 5-methylcytosine to thymine, and functions via an endonuclease that nicks the DNA at T:G mismatches in sequences methylated by DNA cytosine methyltransferase, and does so in a strand specific way (58, 60). The resulting gap is filled and completed by DNA polymerase and ligase.

The MMR system is very conserved and is the main repair complex that follows the replication fork to correct bases that escaped Pol III editing, increasing replication accuracy by 20-400 fold (56). In eukaryotes and most bacteria, MMR functions by recognizing strand discontinuities; the mismatched basepair is recognized by the MutS(α) protein, followed by a strand nick by the MutL(α) protein on the discontinuous strand and removal of the patch of DNA containing the erroneous base by an exonuclease (56, 61). Contrastingly, *E. coli* uses methylation signals for strand discrimination; MutS recognizes the mispair and MutL binds to form a complex, then recruiting MutH to make an incision at a nearby unmethylated GATC on the newly synthesized strand. A helicase then unwinds the DNA patch and exonucleases remove the strand containing the incorrect base (62). Lastly, in all cases, DNA Pol δ/III and DNA ligase fill in and complete the repair (56, 61).

Lesions in the DNA that result in single- or double-strand breaks block the progression of replication, and these breaks are repaired by recombination repair using a homologous segment of DNA within the same cell as a template (63). First, DNA ends are processed to produce 3' single stranded tails. Next, a so-called "joint molecule" is created through strand exchange between one of these 3' ends and a strand of an intact homologous template of DNA. Using

both strands of the intact homologous region, each processed 3' end is elongated to produce the complementary sequence. Lastly, the Holliday junctions are resolved using endonucleases (63-65).

In the event of DNA lesions that block DNA replication, the replication machinery resumes past the lesion, leaving unfilled gaps in the DNA. When these gaps are not resolved, the SOS system is induced to express a family of polymerases that are able to replicate across these lesions (23). A more detailed discussion of SOS bypass occurs later in this chapter.

Table 1-2. Summary of cellular DNA repair processes (From (23)**)**

General mode of operation	Example	Type of lesion repaired	Mechanism
Detoxification	Superoxide dismutase	Prevents formation of oxidative lesion	Converts peroxides into hydrogen peroxide, which is neutralized by catalase
Direct removal of lesions	Alkyltransferases	O-6-alkylguanine	Transfers alkyl group from O-6-alkylguanine to cysteine residue on transferase
	Photolyase	6-4 photoproduct	Breaks 6-4 bond and restores bases to normal
	Photolyase	UV photodimers	Splits dimers in the presence of white light
	Oxidative demethylation	1-methyadenine, 3-methylcytosine	AlkB catalyzes the oxidation of the methyl groups, requiring O_2, α-ketoglutarate, and Fe(II), releasing it as formaldehyde
General excision	*uvrABC*-encoded exonuclease system	Lesions causing distortions in double helix, such as UV photoproducts and bulky chemical additions	Makes endonucleolytic cut on either side of lesion; resulting gap is repaired by DNA polymerase I and DNA ligase
Specific excision	AP endonucleases	AP sites	Makes endonucleolytic cut; exonuclease creates gap, which is repaired by DNA polymerase I and DNA ligase
	DNA glycosylases	Deaminated bases (uracil, hypoxanthine), certain methylated bases, ring-III opened purines, oxydatively damaged bases; and certain other modified bases	Removes base, creating AP site, which is repaired by AP endonucleases
	GO system	8-oxodG	The MutM glycosylase removes 8-oxodG from DNA; the MutY glycosylase removes the A from 8-oxodG-A mispairs, leading to re-creation of an 8-oxodG-C pair, and the MutM glycosylase then removes the 8-oxodG
Postreplication	Mismatch repair system	Replication errors resulting in base-pair mismatches	Recognizes newly synthesized strand by detecting nonmethylated adenine residues in 5′-GATC-3′ sequences; then excises bases from the new strand when a mismatch is detected
	Recombinational repair	Lesions that block replication and result in single-stranded gaps	Recombinational exchange
	SOS system	Lesions that block replication	Activates synthesis of DNA polymerases that allow replication bypass of blocking lesion, resulting in frequent mutations across from lesion, and also mutations across from normal bases

Exogenous Factors Leading to Mutations in DNA

DNA base analogs – Chemical modifications to DNA bases occur both through endogenous cell processes as well as resulting from exogenous sources, and these modifications can threaten normal base pairing. It is for this reason that many base analogs are mutagenic through base substitutions. Treatment with 5-bromouracil results preferentially in A:T→G:C transitions (Chapter 4 in this work, (2)). 5-formyluracil treatment results in several base substitutions, the most prominent being A:T→G:C transitions (66). Furthermore, 5-azacytidine induces G:C→C:G transversions, and 2-aminopurine and zebularine induce G:C→A:T transitions (Chapters 3 and 4 in this work, (67, 68)). Additionally, some analogs can induce frameshift mutations, such as 5-bromouracil, 5-azacytidine, and 2-aminopurine (Chapter 4 in this work, (69)).

Alkylating agents – Alkylating gents are a class of molecules that transfer an alkyl group, such as a methyl or ethyl group, to a range of biological molecules, including DNA. This chemical modification thus changes the structure of the biological target, possibly interfering or changing the target's behavior (70). In reacting with DNA, these agents add both simple or complex alkyl groups to the ring nitrogen or extracyclic oxygens of DNA bases, resulting in a range of mutagenic and cytotoxic adducts (31, 32). An example of a very potent alkylating agent is ethyl methanesulfonate, which alkylates the O^6 position of guanine, causing the alkylated G to now mispair with T (23). Given this mutagenic function, alkylating agents have been shown to be teratogenic, genotoxic, and carcinogenic (71). Ironically, several alkylating agents are widely used in chemotherapy, and while effective in killing cancer cells (70), this treatment puts patients at risk for further damaging outcomes. Beyond this intentional use, alkylating agents are also widely found in the environment, from biological byproducts to pollutants (72-74).

Reactive oxygen species (ROS) – ROS are damaging to cellular components, and when damaging DNA free bases or DNA molecules, the results are mutagenic and carcinogenic (see review in next section for cellular interactions). There are numerous exogenous sources that lead to cellular exposure of these compounds, including direct exposure to ROS such as ozone (75), hydrogen peroxide (76-78), and nitrogen oxides (24), as well as indirect sources that interact with cellular machinery to form reactive oxygen species within the cell, such as carbon tetrachloride (79), phenobarbital (80), bleomycin (81, 82), and benzo-α-pyrene (83).

Ionizing radiation – Like alkylating agents, ionizing radiation, such as X- or γ–rays, is commonly used in the treatment of cancer. However, these types of radiation also damage DNA either through direct ionization or indirectly through the production of free oxygen radicals. This treatment produced a variety of DNA lesions, including single- and double-strand breaks, abasic sites, and oxidized bases (84). Additionally, post-irradiation damage can be induced resulting from the attempted repair of lesions created during the radiation exposure, thus resulting in single- and double-strand breaks (85). In the event of incomplete or incorrect repair of these lesions, cytotoxic effects ensue. Additionally, due to the targeting nature of radiation, resulting lesions are often clustered within one-to-two helical turns of the DNA (85). This clustering of lesions proves even more difficult to repair, thus increasing the mutagenic potential of ionizing radiation-induced damage (86, 87). Additional studies have shown that mutagenic frequency is inversely correlated with the distance of DNA lesions (88-90).

Ultraviolet light – Exposure to ultraviolet (UV) light results in genotoxic effects, leading to mutations and cancer (91). There are two types of UV light that reach the earth via sunlight, the longer wavelength UVA (320-400 nm) and shorter wavelength UVB waves (290-320 nm). While

some carcinogenic involvement has been linked to UVA waves, the higher-energy UVB waves have been shown to have a strong carcinogenic effect on skin (20, 92, 93). UV-irradiation induces photoproducts, including cyclobutane pyrimidine dimers (CPDs), which induce mutations by interfering with base pairing, and pyrimidine(6-4)pyrimidine photoproducts (64PPs) at dipyrimidine sites (23), producing C:G→T:A mutations via the deamination of the cytosine(s) in the lesion (20). Additionally, UV irradiation also produces reactive oxygen species through the activation of molecules like riboflavin, tryptophan, and porphyrin, resulting in oxidative stress (94-97). These reactive oxygen species then induce mutations, as described above and in the next section.

Polycyclic aromatic hydrocarbons (PAHs) – PAHs are lipophilic molecules consisting of two or more condensed aromatic rings. These molecules are produced from the incomplete combustion of organic matter, and sources include forest fires, smoking, vehicle traffic, and crude oil and coal processing (98). PAHs like naphthalene, anthracene, phenanthrene are also commonly used in the production of materials such as dyes, insecticides, and plastics, but in much lower levels than seen from incomplete combustion. Their widespread occurrence along with their carcinogenicity suggest a significant contribution to the incidence of cancer in humans (99, 100). While inert molecules on their own, introduction into mammalian cells results in the activation of these compounds into reactive intermediates that alkylate DNA (101), or into diol-epoxides, which form covalently bonded adducts with biological molecules such as DNA (102). These adducts then result in mutations when subject to misrepair.

Anti-tumor agents – Chemotherapeutic agents are widely used in the treatment of cancer. However, studies have shown that cancer chemotherapeutics are often in fact carcinogenic themselves (21, 103-105). Since chemotherapeutic agents often function through direct or indirect DNA damage, thus preferentially inducing apoptosis in rapidly dividing cells, this

genotoxic activity results in a high mutagenic potential that can result in secondary malignancies (106, 107). Examples of these agents include cisplatin, bleomycin, 5-azacytidine, and zebularine (the latter two discussed earlier, and all four further studied in chapters 2 and 3). Both cisplatin and nitrogen mustards induce DNA-DNA crosslinks, which lead to cell death and inhibiting tumor growth (108, 109). However, these interstrand crosslinks prevent the unwinding of the DNA upon replication (110) as well as result in double-strand breaks. Studies have shown the link between the formation of double-strand breaks and the incidence of mutations and chromosomal translocations (111-116). Bleomycin also forms double-strand breaks, yet indirectly, through the chelation of iron and other metal ions, ultimately generating superoxide and hydroxide free radicals that induce the double-strand breaks (81, 82). Other chemotherapeutic agents such as base analogs induce mutations through direct mispairing, as described earlier.

Cadmium – Cadmium is a toxic and carcinogenic (117) mutagen that exerts it mutagenic activity in a unique fashion. While other mutagens directly induce mutations, and when this occurs at high levels mismatch repair becomes saturated (described later), cadmium instead directly inhibits mismatch repair proteins in yeast and presumably higher cells, thus allowing normal replication errors to go uncorrected, resembling strains deficient in mismatch repair (118, 119). This represents a novel mechanism to be examined in the case of other mutagens.

Endogenous Factors Leading to Mutations in DNA

While exogenous sources of DNA have been extensively studied (as described above), endogenous sources of mutations play a significant role in DNA damage. From byproducts of normal cellular metabolism to DNA replication and repair mechanisms, regular cell mechanisms

from all domains of life produce an extensive set of mutations constantly in battle with repair processes.

Reactive oxygen species (ROS) – In normal cellular respiration, oxygen (O_2) is reduced to water at the end of the electron transport chain. However, within the complexes of the electron transport chain, there is a leakage of electrons that are able to reduce O_2 to the superoxide anion (120). Superoxide is not directly mutagenic, however the free-iron from 4Fe:4S clusters by this anion produces hydroxyl radicals from hydrogen peroxide, and these are the most reactive form of these radicals species (121). Thus, the reduction of oxygen in normal cellular metabolism produces very reactive oxygen radicals that can damage cellular components such as lipids and proteins, and ultimately DNA both directly and indirectly (122), being responsible for a plethora diseases such as cancer, arteriosclerosis, arthritis, and neurodegenerative disorders (123). The discovery of the extent of free oxygen radical involvement in human disease was delayed by the development of techniques to detect oxidative damage in the cell done by these reactive species (124-128). In response to the extensive damage done by reactive oxygen species to cellular components, organisms utilize an broad system of small molecule antioxidants (such as vitamin E and C, carotenoids) to quench the oxidative radical and prevent/minimize the cellular damage (123, 128).

On one hand, direct contact of reactive oxygen species with DNA can produce damaged DNA bases (129), resulting in DNA lesions that are genotoxic and induce mutations commonly seen in mutated human genes, including oncogenes and tumor suppressor genes (123, 130). Activation of certain oncogenes by reactive oxygen species has been demonstrated to occur via point mutations in the K-*ras* and C-*Raf*-1 genes (131). Additionally, free oxygen radical inactivation of tumor suppressor genes (such as p53 and retinoblastoma) has been found to occur via point mutations in CpG dinucleotides (132, 133). Hydroxyl radicals add to the C5 of thymine, C6 of cytosine, and to the C4, C5, and C8 positions of purines. Further oxidation

and/or reduction of these hydroxylated bases yield a plethora of mutagenic base analogs, including the well studied 8-oxo-dG (G:C→T:A) (53, 123, 128), thymine glycol (T:A→C:G), 5-hydroxyuracil, and uracil glycol (134-136). Resulting mutations occur through both misrepair of the lesion as well as replication errors. Additionally, reactive oxygen species can induce strand breaks in the deoxyribosyl backbone, leading to chromosomal rearrangements (129, 137). Furthermore, reactive oxygen species can also interact with nucleotide pools, producing oxidized bases that can then be incorporated into DNA during replication (138).

Beyond direct damage to DNA, the oxygen radicals can oxidize lipids and proteins, which in turn can both alter gene expression or react with DNA, forming adducts (123, 139). The polyunsaturated nature of lipids makes them a vulnerable target to reactive oxygen species, resulting in a chain of free radical reactions (140). Upon reacting with metals, these lipids then form numerous reactive products such as aldehydes and epoxides (123). These reactive products then react with DNA, forming very reactive DNA adducts that are either mutagenic (141) or participate in the formation of DNA-DNA and DNA-protein crosslinks (142), the latter subsequently repaired by the error-prone translesion synthesis (described below).

Additionally, oxidation of proteins in the cell can lead to epigenetic changes that can be oncogenic. Reactive oxygen species stimulate pathways such as protein kinases and *ras* signal transduction pathways that modulate the expression of oncogenes and tumor suppressor genes (143, 144).

Interestingly, studies have found a link between the inflammatory response and cancer (77, 145-147). Conditions involving chronic inflammation, particularly autoimmune diseases such as rheumatoid arthritis, lupus, and vasculitis, have been shown to increase the production of reactive oxygen species. Lymphocytes of patients with these and other conditions have been shown to have increased 8-oxo-dG damage to DNA, along with oxidative damage to lipids, and proteins (148, 149). Furthermore, cells co-cultured with activated phagocytes have been shown to undergo DNA damage, including mutations, strand breaks, and sister chromatid exchange,

resulting in neoplastic transformation (76-78). Researchers suggest that hydrogen peroxide produced by phagocytes cross into nearby cells, and is then transformed into hydroxyl radicals via a Fenton reaction (150). Inflammatory conditions that have been found to be associated with cancer include schistosomiasis, *Helicobacter pylori* infection, asbestos-induced chronic inflammation, chronic hepatitis, and inflammatory bowel disease (146, 151-153).

Recombination events – Homologous recombination is an essential process for the repair of both single- and double-strand breaks. However, studies have shown a link between the resolution of double-strand breaks through homologous recombination and the incidence of mutations and chromosomal translocations (111-116). Furthermore, recombination of homologous sequences can lead to the deletion of important genes, leading to human disease. Deletions between Alu elements result in germ-line diseases resulting from include Tay Sachs (154), Hypercholesterolemia (155-158), and Duchenne's muscular dystrophy (159). Furthermore, these deletions can lead to a variety of cancers (160-167), including human hereditary nonpolyposis colon cancer via the deletion of a human mismatch repair gene MLH1 (160).

SOS bypass – Radman and coworkers and Witkin and coworkers first described the SOS system resulting from studies involving UV irradiation (168-171). Subsequent studies elucidated the genes activated in response to lesions that block DNA replication. Sequence homology divides DNA polymerases into six families, A, B, C, D, X, and Y. *E. coli* has five polymerases: Pol I (A family), Pol II (X family), Pol III (C family), Pol IV (Y family), and Pol V (Y family) (7). Pol I and III are high-fidelity polymerases, and are responsible for the replication of most of the genome. The remaining three polymerases are used in replicating past lesions in DNA, Pol II having a relatively high fidelity, and IV and V being error-prone (7, 172). When normal DNA replication machinery approaches a lesion such as UV photoproducts and bulky adducts, it is

unable to bypass, causing the replication machinery to stop at that point then resume past the lesion. This leaves gaps in the DNA that, when left unfilled, triggers the induction of the SOS system that expresses the error-prone Y family polymerases found in all domains of life (23). Human analogs of these polymerases include Pol K (Pol IV homolog), Pol I, Pol η/XP-V (Pol IV homolog), and Rev1 (172). These polymerases are able to replicate past the lesion, but have a 10-1000-fold lower fidelity than Pol I and III, resulting in a higher frequency of errors across from normal bases (7). Thus, in order to complete and maintain replication, the cell utilizes proteins that put it at a high mutagenic risk. It is for this reason that the cell keeps the intracellular levels of these polymerases tightly controlled at both transcriptional levels, keeping their expression tightly repressed by LexA and expressing them last (Table 1-3 below), and post-transcriptionally, by subjecting the respective protein to rapid degradation by Lon and ClpXP proteases (173).

Table 1-3. Induced SOS genes in *E. coli* and the sequence of their expression (From (174)**)**

Function		Copy number/cell		Heterogeneity
Gene	of gene product	non-induced	induced	index (HI)
Expressed as the first				
lexA	Repressor of SOS genes	1300	7540	6.34;7.02
uvrA	UvrABC-excinuclease (NER repair)	20	250	6.98
uvrB	UvrABC-excinuclease (NER repair)	250	1000	6.11
uvrD	Helicase II	5000–8000	25000–65000	8.80
polB	DNA polymerase II	40	300	12.09
ruvA	RuvAB-helicase,	700	5600	9.19
ruvB	Recombinational repair	200	1600	9.19
dinI	Inhibitor of UmuD processing	500	2300	6.24
Expressed as the second				
recA	SOS derepressor, recombinational repair	1000–10000	100000	4.31
recN	RecN, recombinational repair	?	?	5.16;9.38;11.47
Expressed as the last				
sfiA	(*sulA*) cell division inhibitor	?	125-fold increase	4.65
umuD	UmuD' (unit Pol V)	180	2400	2.77
umuC	UmuC (Pol V)	0	200	2.77

Repair systems immortalizing mutations – As described earlier, oxidized guanines directly mispair with adenine in DNA. The mismatch repair protein MutY is responsible for excising the adenine from the mispair (175). For example, when guanine is oxidized in DNA and an adenine is accidentally incorporated across from it, MutY removes the misincorporated adenine, allowing MutM to repair the lesion and maintain the original sequence (18, 55, 175). However, unlike the mismatch repair MutHLS complex (MMR; described earlier), MutY activity is independent of strand-discrimination. Thus, when 8-oxo-dGTP is misincorporated directly across from adenine in DNA, MutY instead excises the correct adenine, which is later replaced by cytosine. MutY actually facilitates the occurrence of the A:T→C:G transversion (23, 176). Vidmar and Cupples have shown that in a *mutT*-deficient strain, A:T→C:G mutations are enhanced by increasing MutY activity, and reduced when MutY is inactive (176, 177). Additionally, Kim *et al.* demonstrated that MutY competes with the mismatch repair complex in the repair of A:C mispairs. In cells deficient in nucleoside diphosphate kinase (resulting in high dCTP pools and A:T→G:C transitions), the authors observed a sharp decrease in A:T→G:C transitions in the absence of MutY, indicating that MutY itself is responsible for the endogenous transition mutation. The MutY protein always removes the adenine in the recognized mispair, regardless of which strand it is on. Thus if the cytosine is the incorrect base, MutY is immortalizing this mutation. The MMR complex, however, also repairs this mutation, but uses strand discrimination lead by the methylation on the original strand, thus excising the incorrect base (23, 53).

Saturation of mismatch repair – While base analogs and frameshift mutagens like 5-bromouracil (178), 2-aminopurine (179), 9-aminoacridine (180), and oxazolopyridocarbazole (181) induce mutations repaired by mismatch repair, after a certain threshold, these mutations can saturate the mismatch repair complex, thereby allowing other endogenous mutations such

as replication errors to go uncorrected (23). Negishi *et al* demonstrated that in low doses of a base analog mutagen deoxyribosyl-dihydropyrimido(1,2, 4,5-c)(1,5)oxazin-7-one, the frequency of mutations in the mismatch repair-deficient strains (*mutH*, *mutL*, *mutS*) was much higher than in the wild-type strains. However, at high doses the mutation frequencies converged, suggesting a saturation of the mismatch repair complex. Further overexpression of mismatch repair genes in this scenario then drastically reversed the mutation frequency (182). Additionally, studies have shown that the hypermutability resulting from the disruption of the Polymerase III epsilon editing subunit is significantly greater than the fidelity the editing function contributes to the polymerase, further suggesting a partial saturation of the mismatch repair system resulting from excess errors resulting from a lack of proofreading ability (183-188). Furthermore, Miller *et al*. demonstrated that frameshift mutations resulting from mismatch repair saturation are produced indirectly. After treatment of cells with the mutagen ethyl-methanesulfonate (EMS), the resulting lesion normally recognized by the MutS protein, the cells become deficient in MMR phenotypically. Thus, after the introduction of an unmutagenized target in cells, the authors observed a 40-80-fold increase in frameshift mutations at repeat tract sequences, revealing that mutations normally repaired by MMR persist even after the cell is mutagen-free (189).

Nucleotide repeat sequences – Nucleotide repeat sequences are unstable and very vulnerable to mutation ((190, 191), also called dynamic mutations), and tandem repeats of 3bp→50bp are the sources of many human diseases, from neurodegenerative diseases to cancer (11, 23, 192). When these repeats are located directly upstream or within the coding region of a gene, the number of repeats can affect the transcription level or final transcript product. For each disease there is a normal copy number range, and expansion above this range leads to a disease prognosis. The expansions are either heritable or caused by somatic mutation within the individual (193). One type of tandem repeat commonly associated with disease is the triplet repeat, and the expansion of these repeat sequences have been shown to

cause Huntington's disease, Fragile X syndrome, and Myotonic dystrophy (193). In Fragile X Syndrome, a CGG triplet repeat in the 5' untranslated region (UTR) of the *FMR1* gene undergoes expansion to over 200 repeats, which results in the suppression of gene transcription (194). In Huntington's disease, the CAG repeat is located within the coding region of the *IT-15* gene, and the expansion of this repeat results in disease pathology (195), and Myotonic dystrophy results from the expansion of the CTG repeat in the 3' (UTR) of the *DMPK* gene (196). However, tandem repeat sequences of longer basepair length are also associated with disease states, such as the 24bp tandem repeat sequence in the coding region of the prion protein resulting in Creutzfeldt-Jakob disease (also known as Mad Cow Disease) (193). Short frameshift mutations in simple tandem repeats that result in changes in the repeat number frequently occur as replication errors (called slipped strand mispairing, (23, 197)), and these repeats have been recognized as mutational hotspots (198). However, due to the large jump in repeat numbers seen in tandem repeat disorders, as well as the larger size of some repeats (>20bp), this suggests an alternate mechanism of mutation that involves secondary structures, such as hairpins or loops, which then fold back and allow the looped region to be replicated twice, resulting in an expansion (199).

Contingency loci are another form of repeat tract sequences that provide genetic variability. These loci are often found within the coding or promoter regions of surface antigens for which variability provides a selective advantage, as seen in *Haemophilus influenzae*, *Bordetella pertussis*, and *Escherichia coli* (200-202). See Table 1-4 for a list of bacteria and types of hypermutable repeats used for adaptation (From (202)).

Table 1-4. Hypermutable repeats within various bacteria (From (202)**)**

Genus	Nucleotide composition	Location of repeat unit[a] (no of repeats)	Gene	Function of repeat unit
Yersinia pestis	5′-A-3′	Within ORF (9)	*yadA*	Membrane protein
Campylobacter jejuni	5′-C- 3′	Within ORF (8)	*wafN*	Lipo-oligosaccharide synthesis
Bordetella pertussis	5′-C-3′	Promoter (15)	*fimB*	Adhesin
Helicobacter pylori	5′-CT-3′	Within ORF (8)	HP722	Membrane protein
Haemophilus influenzae	5′-TA-3′	Promoter (9)	*fim A, B*	Pilus
Escherichia coli	5′- TCT-3′	Within ORF (5)	*ahpC*	Stress response
Mycoplasma gallinarium	5′-GAA-3′	Promoter (12)	pMGA	Adhesin
Moraxella catarrhalis	5′- CAAC-3′	Within ORF[b]	*tbp*	Unknown
Neisseria gonorrhoeae	5′-CTCTT-3′	Within ORF (7)	*opa*	*adhesin/invasin*
Haemophilus influenzae	5′-GACGA-3′	Within ORF (4)	*hsd*	Restriction- modification
Haemophilus influenzae	5′-ATCTTTC-3′	Promoter (16)	*hmw*	Adhesin

[a]The number in parenthesis indicates a typical number of SSR repeat units although these vary through slippage.
[b]No data are available for the number of SSR repeat units.

Diversity-generating retroelements – Up until this point endogenous processes that result in deleterious mutations have been described. However, many simpler organisms utilize mutagenic to instead confer a fitness advantage, such as employing the highly error-prone process of reverse transcription (203). Viruses utilize this characteristic to their benefit to produce a wide variety of mutants that can escape host defenses (204). However, a high frequency of mutagenesis can also lead to mutational decay (205). Because of the deleterious nature of most mutations, organisms that use reverse transcriptase have evolved to direct this activity to certain areas of their genomes where hypermutability is favorable. For example, the *Bordetella* bacteriophage takes advantage of the error-prone reverse transcriptase in encoding the exposed part of its tail fiber protein (called the Variable Repeat, or VR), which recognizes and binds to its host (206). In this mechanism, a nearby downstream copy of the tail fiber gene, named the Template Repeat (TR), is expressed and then reverse transcribed in an error-prone

manner. This mutated cDNA then replaces the VR, resulting in an altered tail fiber protein. This isolated variability in the tail fiber allows the bacteriophage more flexibility to recognize and bind to its host, while maintaining the integrity of the rest of its genome (206). Many organisms have now been found to use this mechanism, including *Treponema denticola* (a bacterium in the human mouth), *Legionella pneumophila*, *Nostoc* and *Trichodesmium* species (206).

Global Pathways Involved in Mutagenesis

While the direct mechanism of mutagenic events are mostly understood, less understood are global cell processes that contribute, or that are even directly responsible for the resulting mutagenic event. For example, earlier studies have shown a link between cell division and induction of mutations, leading to cancer (207, 208). A review by Henderson and colleagues noted that carcinogens, specifically which are not directly genotoxic, lead to an increase in cell proliferation. The authors further noted that chemicals administered at a high dose have a certain degree of toxicity, thus promoting overcompensating proliferation in the surviving cells. Notably, the cancers induced by the carcinogens discussed in this study occur more often later in life. Given the numerous genetic alterations usually needed for the development of cancer, this further links cell division to malignancies (208). Gold and coworkers noted that induced cell division multiplies the incidence of DNA damage leading to mutation. The authors suggest the mechanism by which cell proliferation induces mutations relates to the time lag needed to induce DNA repair mechanisms to mutations, and rapid cell division decreases this window. Other explanations for this phenomenon could include increased cellular metabolism demand under rapid proliferation, leading to increased oxidative DNA damage. Genomic studies are still needed to further elucidate this link.

Other studies have linked folate metabolism to DNA stability and integrity. This is of particular interest since epidemiological studies have demonstrated a link between folate

deficiency and cancer incidence, including colorectal, breast, stomach, and pancreatic cancer (209-212). Neurological disorders have also been linked to folate deficiency (213). Folate deficiency has been shown to induce uracil misincorporation and hypomethylation (214). Since folates are required for the biosynthesis of thymine, depletion of these pools give a competitive advantage to uracil in DNA incorporation. Additionally, folate deficiency leads to the increase in chromosomal fragile sites, which are especially susceptible to DNA breakage (215). Many oncogenes and tumor suppressor genes have been found on fragile sites, making these breakages much more dangerous (216). Uracil incorporation has been implicated in hypomethylation and interference of DNA-protein interactions, resulting in the collapse of the chromosome structure (217). However, studies have shown that and excessive folate intake has also been linked to an increase in tumorigenesis (218). This paradox reveals a gap in the understanding of the role of folate in cancer that should be further elucidated.

Interestingly, studies have shown that folate deficiency induces nucleotide pool imbalance (219), and it has long been established that nucleotides pool imbalance results in increased mutations in both bacterial and mammalian cells (219-222). More recent studies have demonstrated that increasing the nucleotide pools also results in a mutator phenotype (223, 224). A more extensive discussion on this topic occurs in Chapter 4.

Furthermore, recent studies have shown the involvement of efflux pumps in the incidence of mutagenesis. Yang *et al.* used a multicopy cloning approach to overexpress *E. coli* genomic fragments to identify mutator genes. They discovered that the overexpression of a negative regulator of multi-drug efflux pumps induced frameshift mutations (225). Furthermore, Gabrovsky *et al.* demonstrated that the overexpression of the *emrR* functional counterpart from *Pseudomonas aeruginosa* also induced both frameshift and base substitution mutations when expressed in *E. coli* (226). These results strongly suggest that the repression of efflux pump expression reduces the cell's ability to eliminate endogenous mutagenic compounds within the cell, possibly intermediates of nucleotide metabolism or damaged bases (23). Other studies

have suggested that efflux pumps function to eliminate toxic metabolic intermediates (227-229). Further studies are required to determine specific metabolites involved in this process.

In the remaining chapters, I will explore these global pathways and how they affect genotoxicity, both induced by DNA-damaging agents and spontaneously. First, I will discuss global pathways that were found to be responsible for the cell's resistance to the cytotoxicity of six chemotherapeutic agents and mutagens. Interestingly, folate biosynthetic pathways were found to be involved in the cell's resistance to a cytidine base analog. Then, I will review pathways that were discovered to contribute to the mutagenicity of two base analogs. These studies uncovered a pathway implicating ribonucleoside pools and RNA degradation in spontaneous mutagenesis, and I will explore the three possible models through which this occurs.

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CHAPTER 2: Determination of Hypersensitivity to Genotoxic Agents Among *Escherichia coli* Single Gene Knockout Mutants

Summary

We have tested the KEIO collection of 3985 different viable single gene knockouts in *Escherichia coli* to identify genes whose loss increases sensitivity to one or more of six different chemotherapeutic agents and mutagens: Bleomycin (BLM), Cisplatin (CPT), ICR-191 (ICR), 5-azacytidine (5AZ), Zebularine (ZEB), and 5-bromo-2'-deoxyuridine (5BdU). We discovered a set of 156 strains that display a significant increase in sensitivity to at least one of the agents tested. Each genotoxic agent generates a distinct "sensitivity profile" that is characteristic of the agent. Comparison with an independent study of sensitivity profiles for an extensive set of antibiotics pinpoints those effects that are relatively specific for each agent. In some cases engineered double mutants have greatly increased effects. These results provide insight into the mechanism of action of each agent, and define targets for the design of co-drugs that can potentiate these agents. An example is the finding that mutants lacking one of several genes in the folate biosynthetic pathway are hypersensitive to ZEB, leading to a demonstration of synergy between trimethoprim and ZEB.

Introduction

Bacteria have often served as model systems for the interaction of genotoxic agents with DNA, and for analyzing the effects of mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and recombinational repair systems on DNA lesions, as exemplified by studies with cisplatin (1-9), leading to similar studies of DNA repair of such lesions in higher cells (5, 10-14). In this study we sought mutants in *Escherichia coli* that have increased

sensitivity to one of six or more genotoxic agents (bleomycin (BLM), cisplatin (CPT), ICR-191 (ICR), 5-azacytidine (5AZ), zebularine (ZEB), and 5-bromo-2'-deoxyuridine (5BdU)), to define the cell's intrinsic resistance to them, the intrinsic resistome, and to uncover unexpected pathways involved in sensitizing cells to one or more of these agents. Some of these pathways might have counterparts in higher cells and thus prove useful in sensitizing human cells to lower doses of these agents. This would reduce the toxic side effects associated with drug treatment (12, 15), as well as the secondary tumors resulting from the mutagenic effects of the agents themselves. Moreover, the increased potency of the drug could retard the development of resistance that often limits treatment (16).

The six genotoxic agents, BLM, CPT, ICR, 5AZ, ZEB, and 5BdU (see Figure 2-1) that we used to screen the KEIO collection of gene knockouts in *E. coli* for hypersensitivity are both cytotoxic and mutagenic in *E. coli*. However, they are also used in various cancer chemotherapeutic programs, and in some cases are linked to human disease. BLM exerts its effects by chelating iron and other metal ions, ultimately generating superoxide and hydroxide free radicals that cause double-strand breaks in DNA. These are cytotoxic if unrepaired (17, 18). BLM is part of effective regimens, often with CPT, for the treatment of testicular cancer (19), and also for Hodgkins lymphoma (20). CPT acts by making inter- and intra- strand cross links in DNA (3, 4, 8), thus interfering with DNA replication, and is used to treat a variety of cancers, being particularly effective against testicular tumors (21-26). ICR (27) is an effective frameshift mutagen in bacterial and eukaryotic cells (28-31). It causes increased killing of repair deficient HCT116 cells compared with repair proficient cells, and has been suggested as a chemotherapeutic compound on that basis (32). 5AZ and ZEB (33) are cytidine analogs that are incorporated into DNA and RNA during replication and transcription. This leads to mutations in bacteria and higher cells (34-37). The anticancer effects of 5AZ (38, 39) and ZEB (33, 40, 41) occur by interfering with the methylation of DNA, thereby reversing the effects of methylation-induced silencing of tumor suppressor genes (38, 39, 42, 43). 5AZ (38) and ZEB

(43, 44) inhibit DNA methyltransferase, and ZEB is also a competitive inhibitor of cytidine deaminase (45), an enzyme that reduces the potency of cytidine analogs. Therefore ZEB is used in combination with 5AZ to enhance the antitumor activity of 5AZ (46). 5BdU was found to increase the cytoxicity induced by 1,3-bis-(2-chloroethyl)-1-nitrosourea and cisplatin in human glioma cells (47), and was previously used to sensitize cells to radiation therapy (48). It is worth noting that phagocytes produce 5BdU via myeloperoxidase in human inflammatory tissue, and this may contribute to the link between chronic inflammation and cancer (49).

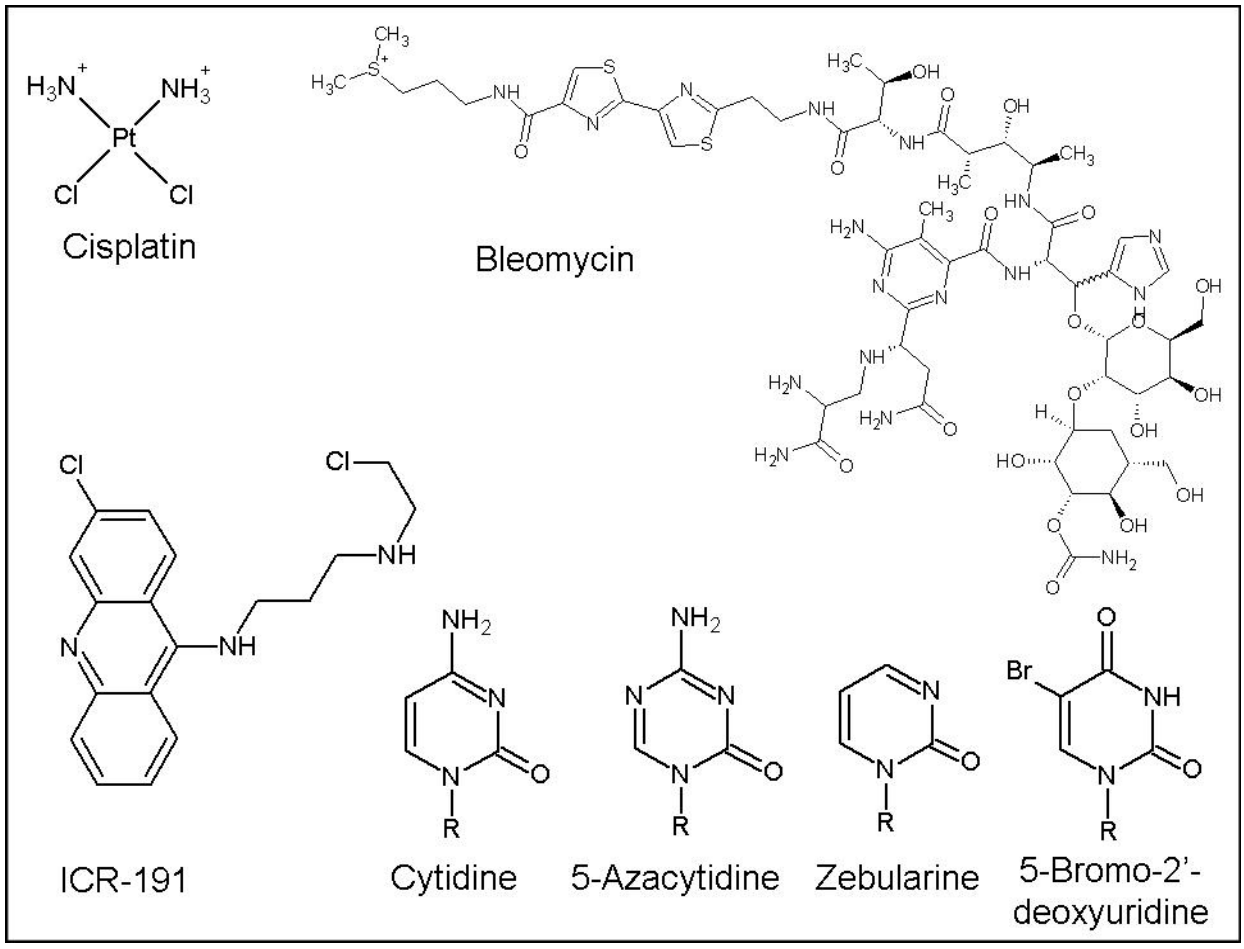


Figure 2-1. Structures of genotoxic agents studied. The "R" group on the base analogs represents the ribose ring, which is in the deoxyribonucleotide form for 5BdU, and in the ribonucleotide form for 5AZ and ZEB (interchangeable for cytidine, used here as a structure reference).

In the work reported here, we screened the KEIO collection of 3,985 mutants (50), each with a different gene knockout, for strains that were more sensitive to one or more drugs, and then quantified the effects by defining the minimum inhibitory concentration, MIC (51). In some cases engineered double mutants have pronounced effects. We detected a number of mutants with altered DNA recombination and repair that have been reported in previous studies (e.g. (1, 2, 7, 9); see Discussion for details), but also found numerous additional mutants affected in functions related not only to DNA, but also cell wall and cell membrane, different metabolic reactions, and gene regulation. We thus have generated a "sensitivity profile" for each agent that represents an additional dimension in agent characterization, namely the response of a comprehensive set of mutants to each chemical.

Materials and Methods

***E. coli* strains –** The KEIO collection is, as described in Baba *et al.* (50), from the starting wild-type strain BW25113 (52). This strain (*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH533}$ *ΔrhaBADL*$_{D78}$) is the wild-type strain used in the experiments reported here, unless otherwise stated. Briefly, each of the 3,985 strains in the KEIO collection carries a complete deletion of a different gene, with a *kan* insert replacing each gene. We used the following strains for donors of the indicated markers by P1 transduction. CGSC6911 – *tolC6::miniTn10* – (*E. coli* genetic stock center); CGSC7553 –*recG265::cat* - (*E. coli* Genetic stock Center); DY330 (53) was converted to *recC::cat* (54) and used as a donor for P1 transduction. N3055 (*uvrA::Tn10*) was a gift of Graham Walker.

***E. coli* genetic methods –** Unless otherwise stated, all genetic methods are as described by Miller (55). P1 transduction was used to generate double mutants in the BW25113 background.

Plates with Agents – We prepared LB plates with different concentrations of each agent by making a fresh stock solution (1 mg/ml CPT, BLM, ICR, ZEB, & 5dBU in sterile distilled water, and 24 mg/ml 5AZ in DMSO), dissolving for at least one hour, and adding to the agar just prior to pouring. All plates were used within several hours of preparation.

Screening with the Duetz cryoreplicator – The Duetz cryoreplicator (56) contains 96 prongs on individual springs, allowing its frequent application to frozen glycerol cultures. The KEIO collection (50) is maintained on two sets of 45 96-well micro titer plates and stored in glycerol at -80^{o}C. Each set of 45 plates contains one copy of the entire collection. We used the odd numbered plate set (plates 1,3,5,7…etc). A Duetz cryoreplicator was used to transfer cells from the frozen plates to 0.5ml media in 96-well plates, which were incubated overnight and then the replicator was used to transfer a microdrop to microtiter wells with fresh LB medium containing 50 μg/ml kanamycin, to prevent the growth of contaminants. After 3 hours of growth these plates were printed onto LB plates with different concentrations of each agent. For the initial screening, kanamycin was present in the plates, but in final retests and MIC determinations kanamycin was not present. Initially, 200 μg/ml of CPT, 0.3 μg/ml BLM, and 20 μg/ml ICR was used to screen all 45 plates in the collection. (Figure 2-2A shows an example). The MIC (minimum inhibitory concentration) for the starting strain (BW25113) is 250 μg/ml for CPT, 1.25 μg/ml for BLM, and >30 μg/ml for ICR. Strains appearing more sensitive to one of these agents were retested at 50, 100, 150, 200, and 250 μg/ml CPT, 0.125, 0.313 and 0.5 μg/ml BLM, and 10, 15, and 20 μg/ml ICR. We created a condensed library consisting of all the sensitive strains as well as 283 strains found to be sensitive to one of 22 antibiotics (57). We tested 5AZ and ZEB against the condensed library of strains at different concentrations (5AZ 60, 80 and 100 μg/ml and ZEB 60 and 100 μg/ml). The MIC of the starting strain was

determined to be >200 μg/ml 5AZ and 150 μg/ml ZEB. Then we tested the condensed library from the first three screens against an array of 5BdU concentrations (50, 100, 200, and 300 μg/ml), and the only mutant found to be hypersensitive to 5BU was *dam*. While the starting strain was found to have a MIC of >1000 μg/ml 5BU, the MIC of the *dam* mutant was 50 μg/ml.

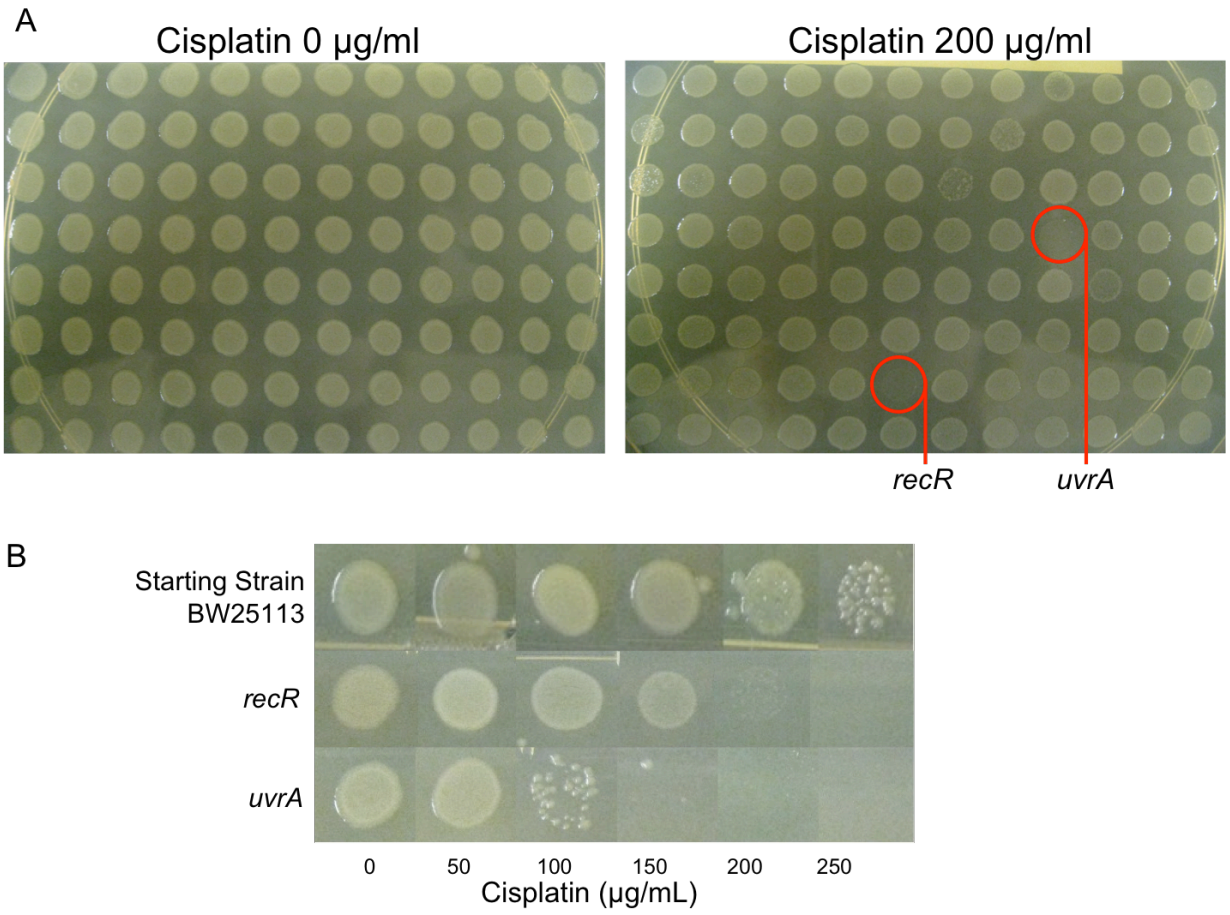


Figure 2-2. Examples of *E. coli* mutants that possess hypersensitivity to cisplatin, as seen in the KEIO collection screen. A) Shown are two 96-well colony grids. On the left are colonies printed onto media containing Luria Broth and Agar only (no drug), and on the right onto media containing a sub-lethal concentration of cisplatin (200μg/ml). Two mutants, *recR* and *uvrA*, do not grow at this concentration, and upon retesting were determined to have minimum inhibitory concentrations of 200 and 100 μg/ml CPT, respectively. B) The growth profiles of *recR* and *uvrA* that show significant sensitivity to cisplatin with respect to the starting strain (BW25113) are illustrated in order of increasing sensitivity. The figure is a composite of printed colonies (see Materials and Methods) of each strain on media with different concentrations of CPT.

Determination of MIC – Minimum inhibitory concentrations (51) were determined either by applying 10^4–10^5 colony forming units to an LB plate with the appropriate concentration of the desired antibiotic and examining the plate after 18 hr incubation at 37°C, or by inoculating liquid

cultures with 10^5 cells and observing growth after 18 hours (51). Most of the mutants in the KEIO collection grow indistinguishably from the wild-type on LB plates under the conditions tested.

Combination experiments with zebularine and trimethoprim – The starting strain was grown over night in minimal medium (Medium A (55)), then sub-cultured for 6 hours, also in minimal medium until a density of ~10^8 cells. The subculture was then diluted 10^{-2}, and 50μl of the dilution was added to 5ml of minimal media with or without each agent. Cultures were incubated at 37°C for 18 hours, and then the optical density of each culture was taken at 600 nm. First, concentrations of ZEB and trimethoprim (TMP) to be used were determined (>70% growth for each drug alone as compared to the control). A concentration of 0.03 μg/ml TMP and two concentrations of 0.04 μg/ml and 0.05 μg/ml ZEB were determined. For the experiments, a control (no drug), each drug alone, and TMP in combination with both ZEB concentrations were all tested in triplicates.

Colony Density Analysis – 96-well colony grids were photographed using a Canon 8.1 megapixel camera. The images were then cropped before analysis. Density Analysis of the colonies was performed using software ImageJ (http://rsbweb.nih.gov/ij/). First, background removal was performed uniformly across every image, and then the contrast for each was increased uniformly by 2%. The raw values obtained from this program were then input into excel, in which the raw values of the two control strains, *ebgA* and *bglB*, were subtracted from the values of all other colonies. The absolute values resulting from this indicate the level of sensitivity, higher values being most sensitive and vice versa. These adjusted values were then combined into a single chart, which was then condensed by setting a cutoff value of 7,000 for each genotoxic agent tested. However, these values only account for one concentration of

each agent, and thus do not give the same rank-order information as is given by visual inspection, though still providing accurate quantitative sensitivities. Additionally, mutants that only show growth of revertant colonies yield lower sensitivity values due to a strong signal from their dense growth. This phenomenon, as well as the high cut-off values chosen for these values in an attempt to minimize noise, is responsible for lower number of hits in this table (Figure A2-2) when compared to Figure 2-3 (described in Figure A2-3), although all the hits from this method were found via visual inspection.

Validation of strains – A collection of 3,985 strains will contain some errors, and some impure strains. Mori and coworkers (58) have published a validation of the entire collection, extensively testing each strain, and identifying those that are incorrect or are suspected to be incorrect. We have removed these strains from our charts and tables. The impurity problem can be minimized by repurifying and retesting, as was done here. We also analyzed 22 mutants that were of interest by PCR analysis, and/or sequencing, using two primer pairs for each, and verified in two PCR experiments for each that they all carried a *kan* insert in place of the correct gene, and that there was no presence of the original gene sequence elsewhere (via a duplication). We reported these validations for mutants deleted for *argO, degP, dacA, glpD, trxB, xseA, sapC, rplI, tufA, ycdZ, dinB*, *recA, oxyR, rpsf, flgB, ppiD* (57), and carried out the same analysis for *uvrA, uvrC, udk, umuC, and umuD* in the current study. In the case of *rpsF*, we carried out both PCR and sequencing for validation. An additional two mutants can be identified by their phenotypes (*dam*, mutator; *lon*, muccoid formation). We had reason to suspect the *recF* deletion mutant, and a similar analysis revealed that the *recF* deletion strain in the first copy of the KEIO strains is incorrect, although the *recF* strain in the second copy is correct (57), and this has been used in the work reported here.

Chemicals – Cisplatin, ICR-191, 5-azacytidine, 5-bromo-2'-deoxyuridine, trimethoprim, and kanamycin were purchased from Sigma (St. Louis, MO). Bleomycin was purchased from CalBiochem (San Diego, CA). Zebularine was a gift from Victor E. Marquez.

Results

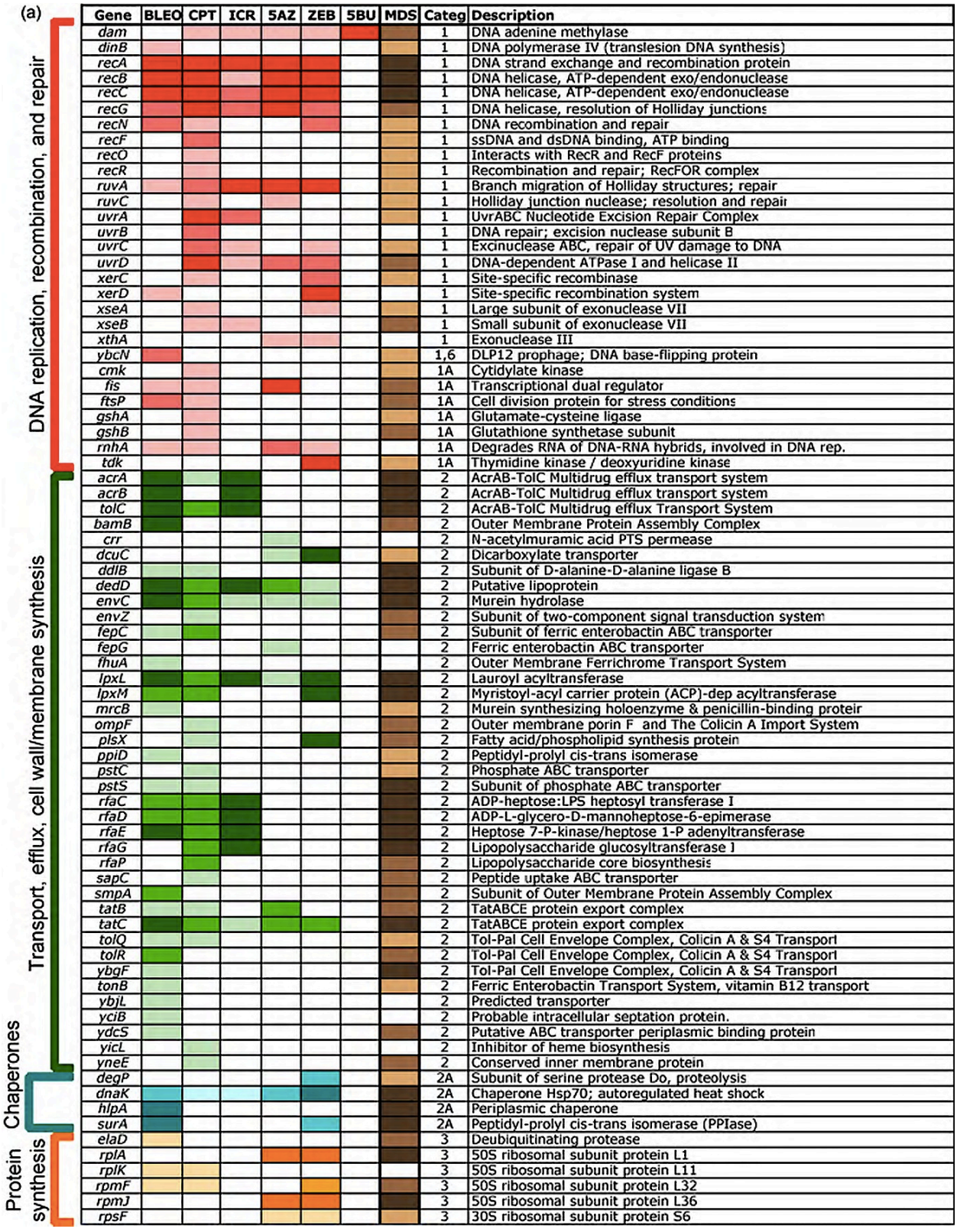
Screening for increased sensitivity to Genotoxic Agents – The entire KEIO collection of 3,985 single gene knockout strains of *E. coli* was screened for mutants with increased susceptibility to CPT, BLM, and ICR (see Materials and Methods). The mutant strains that appeared to have greater sensitivity than the wild-type were purified from single colonies and retested against a set of concentrations for each of the three agents used in the screen, generating a condensed mutant library. This library was added to a condensed library of 283 strains detected by screening for increased sensitivity to any of 22 different antibiotics (57). The condensed library was then tested against 5AZ, ZEB, and 5BdU at varying concentrations. Additionally, 5AZ and ZEB were screened against the half of the KEIO collection that contains most of the assigned genes. Figure 2-2B shows a set of cropped images of mutants grown on different concentrations of CPT to illustrate the varying sensitivities between the strains.

The results from all of the tests are summarized in Figure 2-3, which lists the strains with the highest sensitivity to all six agents using a three-tiered color designation for each data point, the darkest color depicting greatest sensitivity (See Figure A2-1 for the specific MIC values). The sensitivities of the strains listed in this figure were scored by visual inspection, taking their growths across various concentrations into account. Figure 2-3 also compares the data for the six agents with a compilation of the results from our studies of 22 antibiotics tested against the same KEIO collection (57). (Although 283 gene knockout mutants show increased sensitivity to one of the antibiotics tested, we have only considered those mutants that are more sensitive to at least one of the six genotoxic agents tested). This comparison allows us to distinguish

mutants that show sensitivity to multiple antibiotics and genotoxic agents from mutants that show more specific effects, and both types of mutants are evident. The MDS column in Figure 2-3 indicates the degree of multidrug sensitivity for each knockout mutant, with the darkest shade showing the highest degree of MDS, and the empty (white) boxes indicating no increased sensitivity to any of the antibiotics tested. We consider these results in more detail in the Discussion.

Quantifying sensitivity values – Digital photographs of the retested strains were analyzed using colony density analysis software (described in Materials and Methods). To minimize background, sensitivity values were adjusted according to two control strains, and a cut-off value was chosen. The adjusted values above this cutoff were taken and compiled into a list of strains shown in Figure A2-2. All of these strains had been found by visual inspection as described above for Figure 2-3.

Testing double knockout mutants for greater sensitivity – We constructed a number of double knockout strains (see Materials and Methods) to test for further increases in sensitivity to CPT. The results are shown in Table 2-1. It can be seen that in a number of cases the double mutants are considerably more sensitive than either single mutant. For example, *uvrA recC* and *uvrA recG* double mutants display a MIC of 25 μg/ml with CPT, whereas each parent single mutant displays a MIC of 100 μg/ml CPT.



Gene	BLEO	CPT	ICR	5AZ	ZEB	5BU	MDS	Categ	Description
dam								1	DNA adenine methylase
dinB								1	DNA polymerase IV (translesion DNA synthesis)
recA								1	DNA strand exchange and recombination protein
recB								1	DNA helicase, ATP-dependent exo/endonuclease
recC								1	DNA helicase, ATP-dependent exo/endonuclease
recG								1	DNA helicase, resolution of Holliday junctions
recN								1	DNA recombination and repair
recF								1	ssDNA and dsDNA binding, ATP binding
recO								1	Interacts with RecR and RecF proteins
recR								1	Recombination and repair; RecFOR complex
ruvA								1	Branch migration of Holliday structures; repair
ruvC								1	Holliday junction nuclease; resolution and repair
uvrA								1	UvrABC Nucleotide Excision Repair Complex
uvrB								1	DNA repair; excision nuclease subunit B
uvrC								1	Excinuclease ABC, repair of UV damage to DNA
uvrD								1	DNA-dependent ATPase I and helicase II
xerC								1	Site-specific recombinase
xerD								1	Site-specific recombination system
xseA								1	Large subunit of exonuclease VII
xseB								1	Small subunit of exonuclease VII
xthA								1	Exonuclease III
ybcN								1,6	DLP12 prophage; DNA base-flipping protein
cmk								1A	Cytidylate kinase
fis								1A	Transcriptional dual regulator
ftsP								1A	Cell division protein for stress conditions
gshA								1A	Glutamate-cysteine ligase
gshB								1A	Glutathione synthetase subunit
rnhA								1A	Degrades RNA of DNA-RNA hybrids, involved in DNA rep.
tdk								1A	Thymidine kinase / deoxyuridine kinase
acrA								2	AcrAB-TolC Multidrug efflux transport system
acrB								2	AcrAB-TolC Multidrug efflux transport system
tolC								2	AcrAB-TolC Multidrug efflux Transport System
bamB								2	Outer Membrane Protein Assembly Complex
crr								2	N-acetylmuramic acid PTS permease
dcuC								2	Dicarboxylate transporter
ddlB								2	Subunit of D-alanine-D-alanine ligase B
dedD								2	Putative lipoprotein
envC								2	Murein hydrolase
envZ								2	Subunit of two-component signal transduction system
fepC								2	Subunit of ferric enterobactin ABC transporter
fepG								2	Ferric enterobactin ABC transporter
fhuA								2	Outer Membrane Ferrichrome Transport System
lpxL								2	Lauroyl acyltransferase
lpxM								2	Myristoyl-acyl carrier protein (ACP)-dep acyltransferase
mrcB								2	Murein synthesizing holoenzyme & penicillin-binding protein
ompF								2	Outer membrane porin F and The Colicin A Import System
plsX								2	Fatty acid/phospholipid synthesis protein
ppiD								2	Peptidyl-prolyl cis-trans isomerase
pstC								2	Phosphate ABC transporter
pstS								2	Subunit of phosphate ABC transporter
rfaC								2	ADP-heptose:LPS heptosyl transferase I
rfaD								2	ADP-L-glycero-D-mannoheptose-6-epimerase
rfaE								2	Heptose 7-P-kinase/heptose 1-P adenyltransferase
rfaG								2	Lipopolysaccharide glucosyltransferase I
rfaP								2	Lipopolysaccharide core biosynthesis
sapC								2	Peptide uptake ABC transporter
smpA								2	Subunit of Outer Membrane Protein Assembly Complex
tatB								2	TatABCE protein export complex
tatC								2	TatABCE protein export complex
tolQ								2	Tol-Pal Cell Envelope Complex, Colicin A & S4 Transport
tolR								2	Tol-Pal Cell Envelope Complex, Colicin A & S4 Transport
ybgF								2	Tol-Pal Cell Envelope Complex, Colicin A & S4 Transport
tonB								2	Ferric Enterobactin Transport System, vitamin B12 transport
ybjL								2	Predicted transporter
yciB								2	Probable intracellular septation protein.
ydcS								2	Putative ABC transporter periplasmic binding protein
yicL								2	Inhibitor of heme biosynthesis
yneE								2	Conserved inner membrane protein
degP								2A	Subunit of serine protease Do, proteolysis
dnaK								2A	Chaperone Hsp70; autoregulated heat shock
hlpA								2A	Periplasmic chaperone
surA								2A	Peptidyl-prolyl cis-trans isomerase (PPIase)
elaD								3	Deubiquitinating protease
rplA								3	50S ribosomal subunit protein L1
rplK								3	50S ribosomal subunit protein L11
rpmF								3	50S ribosomal subunit protein L32
rpmJ								3	50S ribosomal subunit protein L36
rpsF								3	30S ribosomal subunit protein S6

Gene	BLEO	CPT	ICR	5AZ	ZEB	5BU	MDS	Categ	Description
sirA								3	Peptidyl-prolyl cis-trans isomerase (PPIase)
yfgC								3	Predicted peptidase
yheM								3	Subunit of sulfur transfer protein complex
yheN								3	Sulfur transfer protein, tRNA modification
pcnB								3A	Poly(A) polymerase (Plasmid copy number protein).
pnp								3A	Subunit of polynucleotide phosphorylase and degradosome
rhlB								3A	ATP-dep RNA helicase of the RNA degradosome
rppH								3A	Putative invasion protein
aceE								4	Component of pyruvate dehydrogenase complex
aceF								4	Dihydrolipoamide acetyltransferase
ackA								4	Propionate/acetate kinase
pta								4	Phosphate acetyltransferase
atpG								4	ATP synthase, F1 complex, Y subunit
dapF								4	Diaminopimelate epimerase
fabF								4	Subunit of KASII; fatty acid biosynthesis
fliI								4	Flagellum-specific ATP synthase
folB								4	Dihydroneopterin aldolase
folP								4	Dihydropteroate synthase
nudB								4	Dihydroneopterin triphosphate pyrophosphohydrolase
ygfA								4	Putative ligase, folate biosynthesis
gmhB								4	D,D-heptose 1,7-bisphosphate phosphatase, (lipo biosynth)
gpmM								4	Phosphoglycerate mutase, cofactor independent
iscS								4	Cysteine desulfurase monomer
lpdA								4	Lipoamide, 2-oxoglutarate, & pyruvate dehyd; glycine cleavage
metL								4	Aspartate kinase / homoserine dehydrogenase
nagA								4	Subunit of N-acetylglucosamine-6-phosphate deacetylase
pgm								4	Phosphoglucomutase
pgmB								4	Beta-phosphoglucomutase
rpe								4	Ribulose phosphate 3-epimerase
rpiA								4	Ribose-5-phosphate isomerase A
ubiG								4	bifunctional methylase, ubiquinone biosynthesis
upp								4	Uracil phosphoribosyltransferase
ybgC								4	Esterase/thioesterase
ycjU								4	β-phosphoglucomutase/β-phosphoglucomutase
ydbD								4	Protein involved in detoxification of methylglyoxal
yebR								4	Free methionine-(R)-sulfoxide reductase
ygcO								4	Predicted 4Fe-4S cluster-containing protein
ygfZ								4	Folate-binding protein, RNA modification
yigB								4	FMN phosphatase
ytjC								4	Phosphoglycerate mutase 2
zwf								4	Glucose 6-phosphate-1-dehydrogenase
deaD								5	DEAD-box RNA helicase
dksA								5	RNA polymerase-binding transcription factor
fur								5	Ferric uptake regulation protein.
gcvA								5	Transcriptional dual regulator
hfq								5	RNA-binding protein that affects many cellular processes
nusB								5	Transcription antitermination protein
oxyR								5	Transcriptional dual regulator
rfaH								5	Transcriptional antiterminator
rseA								5	Anti-sigma factor
xapR								5	Transcriptional activator
yciT								5	DNA-binding transcriptional regulator
ymfT								5,6	e14 prophage; DNA-binding transcriptional regulator
zapB								5	Cell division factor
racC								6	Prophage gene and phage related functions
ydfP								6	Qin prophage; conserved protein
ylcG								6	DLP12 prophage; predicted protein
JW3133								7	Hypothetical protein
JW5015								7	Hypothetical protein
JW5115								7	Hypothetical protein
JW5360								7	Hypothetical protein
JW5474								7	Hypothetical protein
ybaB								7	Hypothetical protein
ybeD								7	Hypothetical protein
ybeY								7	Hypothetical protein
ybfJ								7	Hypothetical protein
ybhT								7	Hypothetical protein
ycbK								7	Hypothetical protein
ycbW								7	Hypothetical protein
yceD								7	Hypothetical protein
ychJ								7	Hypothetical protein
yciM								7	Hypothetical protein
yddK								7	Hypothetical protein
yfiH								7	Hypothetical protein
yhcB								7	Hypothetical protein
yidD								7	Hypothetical protein
yjjI								7	Hypothetical protein
yjjY								7	Hypothetical protein

Figure 2-3. Top Sensitive *E. coli* Strains to genotoxic agents. 156 single gene knockout mutants were found to be hypersensitive to one or more genotoxic agents. Here strains are organized first by gene category (1(red) - DNA replication, recombination and repair, 1A(red) - functions indirectly affecting category 1, 2(green) - transport, efflux, cell wall and cell membrane synthesis, 2A(teal) - chaperones and functions related to 2, 3(orange) - protein synthesis, 3A(orange) - RNA processing-7(black) - unassigned genes), then alphabetically, though some strains involved in the same pathway are grouped together. Strains with stronger susceptibilities are shown in the darkest shade, medium susceptibilities in medium shade, and weak susceptibilities in lightest shade. Column 5 (MDS) indicates the degree of multi-drug sensitivity for each knockout mutant, which we determined in a separate study (57), with the darkest shade showing the highest degree of MDS (sites sensitive to 8 or more agents), the medium shade 4-7, the light shade 1-3, and the empty (white) boxes indicating no increased sensitivity to any of the antibiotics tested.

Combination treatments – One of the objects of this work is to detect pathways or proteins that when inhibited might increase the sensitivity to a particular agent. There are four mutants, *ygfA, folB, folP,* and *nudB*, defective in different reactions in the folate biosynthetic pathway that are more sensitive to one or more of the agents tested. The hypersensitivity of three of these to ZEB led us to examine whether a combination of trimethoprim (TMP), an inhibitor of dihydrofolate reductase, and ZEB would show synergistic effects. Figure 2-4 displays the results using ZEB and TMP in combination against the starting strain, BW25113, which clearly show that concentrations of TMP and ZEB that give little growth inhibition by themselves result in virtually a total inhibition of growth when used in combination.

Table 2-1 - Minimum Inhibitory Conentrations (MICs) in Single and Double *E. coli* Knockout Strains

Strain	MIC (CPT, µg/ml)	Double Mutant	MIC (CPT, µg/ml)
BW25113	200-250		
uvrA	100		
recG	100	*uvrArecG*	25
recC	100	*uvrArecC*	25
recO	150	*uvrArecO*	50
tolC	150	*uvrAtolC*	100
gcvA	150	*gcvArecC*	50

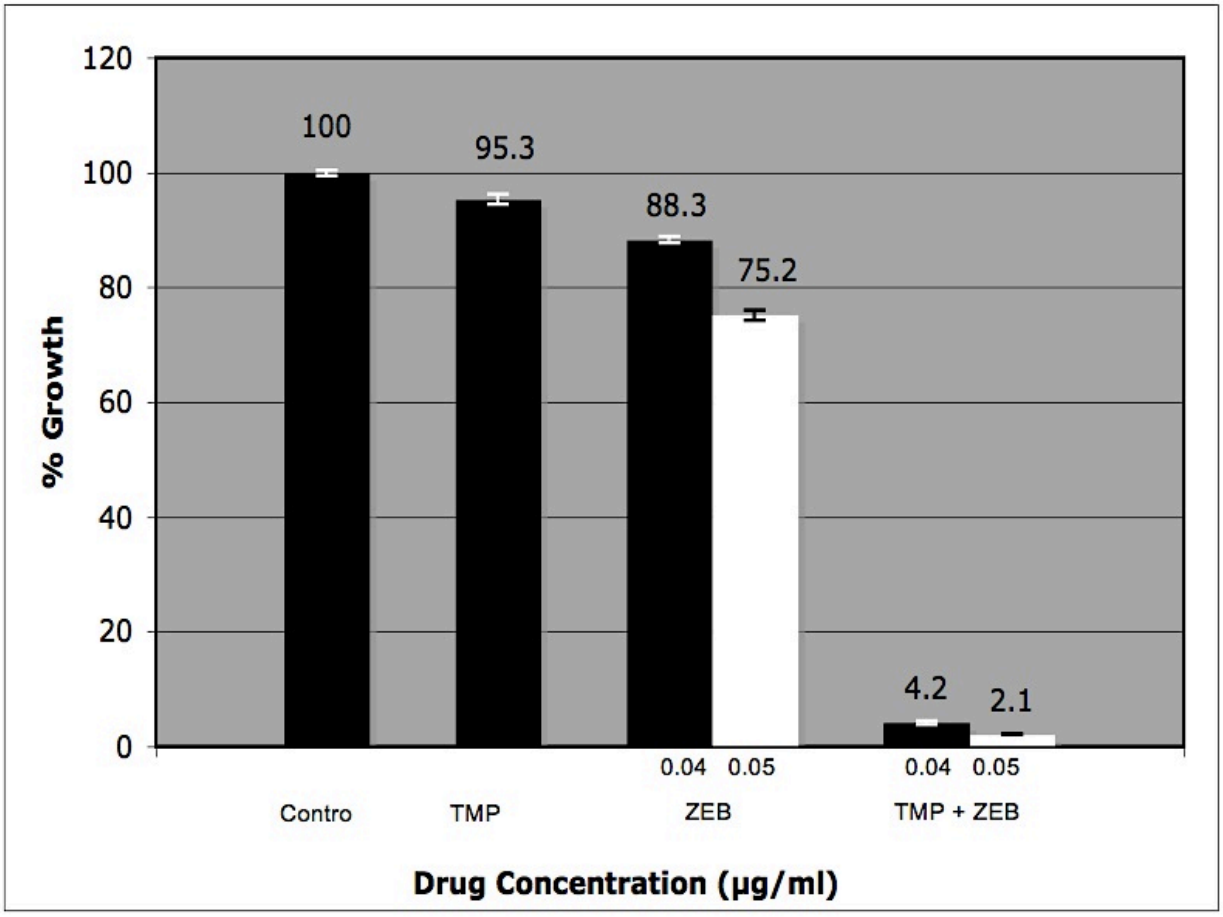


Figure 2-4. Combination experiments between zebularine and trimethoprim. The hypersensitivity of folate biosynthesis mutants to ZEB led us to examine whether a combination of trimethoprim (TMP), an inhibitor of dihydrofolate reductase, and ZEB would show synergistic effects, using the starting strain (BW25113). This figure shows that concentrations of TMP 0.03μg/ml and ZEB 0.04 and 0.05μg/ml that give little growth inhibition by themselves result in virtually a total inhibition of growth when used in combination.

Discussion

We chose to look at mutants that are more sensitive to at least one of six genotoxic agents, CPT, BLM, ICR, 5AZ, ZEB, and 5BdU, in order to uncover pathways of damage avoidance and repair not yet described for these agents, and to define the total "intrinsic resistome" for each compound. We screened the KEIO collection (50) of 3,985 single gene knockout *E. coli* strains and after retesting defined a set of 156 mutants with increased

sensitivity to one or more of the agents. Figure 2-3 lists these mutants according to the function affected. Previous work on mutants sensitive to these agents has focused on those deficient in DNA recombination and repair. CPT, BLM, and 5AZ have been well studied, ICR and 5dBU less so in this regard, and ZEB virtually not at all. Marinus, Essigmann, and coworkers (2, 6, 9), among others (1, 4), have shown that mutants deficient in the *recA,B,C* pathway, *recF*, and nucleotide excision repair deficient mutants such as *uvrA*, are more sensitive to CPT than wild-type, as are *dam* mutants (3). Some of these sensitivities emanate from CPT-induced DNA double-stranded breaks (59). Additional work from Marinus and Essigmann (60) showed that *recA,B,C,D,* and also *recN, recG*, and *ruvC,* but not *recF*, are more sensitive to BLM than wild-type. The results in Figure 2-3 are in good agreement with these findings, particularly considering that different types of measurements have been used. Ikeda and coworkers (61) have reported moderately increased sensitivity of *hns* mutants to BLM at 30°C, but we have not detected sensitivity in our experiments at 37°C. An extensive study of 5AZ sensitivity (62) examined 25 repair and recombination mutants, and detected *recA,B,C,G, ruvA,C, uvrD* mutants as being significantly more sensitive than wild-type. Again, the results in Figure 2-3 are in agreement with these findings. They also detected sensitivities with *priA, priB,* and *polA* mutants. Valid *priB* and *polA* mutants are absent from the KEIO collection (58). Strains lacking adenine methylase, *dam*, have been shown to be sensitive to base analogs such as 2-aminopurine, and 5-bromouracil, and attributed to the generation of MMR-dependent double strand breaks (63). Finally, Exo VII has been shown to be involved in mutation avoidance by acridines (64).

With regard to DNA repair, new results shown in Figure 2-3 include the involvement of Exo VII in protecting the cell from CPT and ZEB, as strains lacking either subunit (*xseA,B*) are more sensitive to CPT and those lacking at least the large subunit (*xseA*) are more sensitive to ZEB. Also, Exo III (*xthA*) protects against the toxicity of both 5AZ and ZEB. The novel finding

that *rnhA* mutants are more sensitive to BLM, CPT, 5AZ, and ZEB raises the question of how an enzyme that degrades RNA of DNA-RNA hybrids protects the cell from these agents. The involvement of *recA,B,C,G,N, ruvA, uvrC,D,* and *xerC,D* in protecting the cell from the toxic effects of ZEB are also revealed in Figure 2-3. New roles for detoxifying enzymes are exemplified by the finding that ICR has greatly increased cytotoxicity in *ybdB* mutants lacking a detoxifying protein for methylglyoxal.

Figure 2-3 shows that each of the six genotoxic agents has a distinct "sensitivity profile". Some of the mutants are also sensitive to multiple antibiotics (57). We indicate these in the MDS (multiple drug sensitivity) column. These mutants have defects in one of a number of different functional categories, including recombinational repair (*e.g. recABC*), efflux (*acrAB, tolC*), membrane and cell wall synthesis and maintenance (*rfaCDEG, envC, lpxLM*), and control of gene expression (*nusB, rseA, xapR, yciT*). The more specific sensitivities are of greater interest here, and they are also distributed among each functional category. Twenty-five of the mutants are not sensitive to any of the antibiotics tested (57) but are sensitive to one of the six genotoxic agents studied here.

Although 5AZ, ZEB, and 5BdU are base analogs, their sensitivity profile fingerprints are completely different. Only one mutant, among those tested, namely the *dam* knockout, is more sensitive to 5BdU, with a MIC of 50 μg/ml. Even at 1,000 μg/ml, no other mutants appear to be sensitive. 5BdU is in the deoxyribonucleoside form, whereas 5AZ and ZEB are in the ribonucleoside form. Although this might explain the lack of sensitivity of some of the other strains to 5BdU, it does not explain the marked difference between 5AZ and ZEB in these experiments (*e.g.* see the different responses of *xerCD, tdk* and *fis* to these two agents).

Among the relatively specific mutants, several are noteworthy. *yebR* mutants lack free methionine-(R)-sulfoxide reductase, and are strongly sensitive only to ZEB (Figure 2-3), and not to any of the 22 antibiotics tested. The same is true for *ydfP* and *yhcB.* Several mutants defective in the folate biosynthesis pathway are sensitive to BLM, CPT, and particularly ZEB.

Actually, there are 13 genes involved in the multi-step branched pathway for folate synthesis in *E. coli*. Seven of these are deleted in viable mutants in the KEIO collection, and four of these *ygfA, folB, folP,* and *nudB* show sensitivities to genotoxic agents. We have grouped these in Figure 2-3. *upp* (uracil phosphoribosyltransferase) mutants are sensitive to only 5AZ and ZEB. *ybcN* mutants, lacking a prophage encoded base flipping protein, are sensitive to only BLM among the agents tested here, and only rifampicin among the 22 antibiotics tested (57).

Kishony and coworkers looked at effects of combinations of two different antibiotics for a series of antibiotics (65). They found that TMP showed synergistic effects with aminoglycosides and with sulfamonomethoxine, but not with 13 other classes of drugs. The synergistic effects we detected between ZEB and trimethoprim, an inhibitor of bacterial dihydrofolate reductase, shown in Figure 2-4, underscore one of the objects of this study, which is to pinpoint ways to potentiate currently used drugs by identifying pathways of sensitivity. These results suggest possible synergy between ZEB and methotrexate, a related inhibitor of human dihydrofolate reductase that is used in chemotherapy (66).

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CHAPTER 3: Identification of Cellular Targets Involved in 5-Azacytidine-Mediated Mutagenesis in *Escherichia coli*

Summary

5-Azacytidine (5AZ) is a base analog and demethylating agent widely used in the treatment of cancer. While it is an effective chemotherapeutic, it is also mutagenic, which can lead to secondary malignancies. However, the molecular mechanism by which this mutagenesis occurs is not fully understood. To discover what proteins are involved in the production of 5AZ-mediated mutagenesis, we screened a collection of 3,985 *Escherichia coli* single gene knockout mutants, using a papillation assay that detects levels of mutations in each strain. We discovered four mutants involved in the nucleotide salvage pathway that show decreased levels of mutagenesis, implicating RNA turnover and competing nucleotide pools in exacerbating 5AZ-induced mutagenesis.

Introduction

5-Azacytidine (5AZ) is a cytidine base analog (Figure 3-1) first synthesized by Piskala and Sorm (1). It inhibits DNA methylation in both prokaryotes and eukaryotes, and is thus useful in the treatment of cancer (2-5). Hypermethylation of CpG islands within the promoter region of tumor suppressor genes (such as those involved in DNA repair and signal transuction) results in silencing these genes and has commonly been associated with cancer (6, 7). However, studies have shown that these methylated regions can be reactivated by 5AZ ((8) and references therein). 5AZ inhibits DNA methylation by forming a covalent complex with the DNA methyltransferase enzyme, inhibiting this enzyme irreversibly (9, 10), and thus reversing the

methylation-induced gene silencing (2, 5, 11, 12). As a result, this analog is widely used in the treatment of cancer (8).

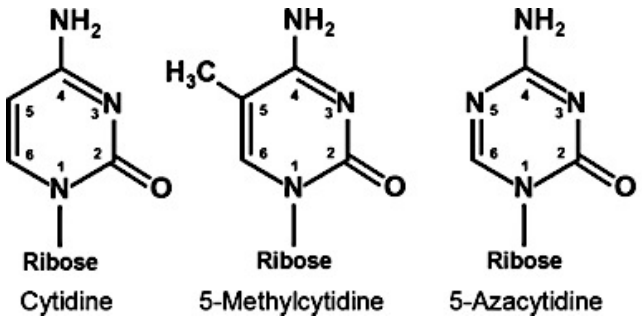


Figure 3-1. Chemical structure of cytidine and its analogs.

5AZ enters the cell via nucleoside transporter proteins (13, 14). Once inside, uridine-cytidine kinase catalyzes the first step in converting 5AZ into an active nucleotide, creating the monophosphate (5, 14, 15). Cytidine deaminase competes with this activation, deaminating 5AZ into an inactive 5-aza-uridine nucleoside (14). Being a ribonucleoside, 5AZ is phosphorylated and preferentially incorporated into RNA, as much as 80-90% in L1210 leukemic cells (16). This incorporation results in cytotoxic effects, such as interfering with RNA processing, ribosome assembly, protein synthesis, and tRNA methyltransferases (17-20). However, a portion of the 5AZ-diphosphate pool is reduced to deoxyribonucleosides by ribonucleoside diphosphate reductase, allowing incorporation into DNA (5). This leads to mutations in bacteria and higher cells (21-25). Studies have demonstrated that 5AZ treatment results in point mutations, specifically C:G→G:C transversions (21, 26, 27), however the mechanism by which this occurs is not completely understood. One theory set forth by Friedman and coworkers is that the 5-azacytosine is converted to the open ring form through hydrolysis of the C6-N1 bond (demonstrated *in vitro* in (28)), thus mispairing with cytosine in the opposite strand (29).

In this chapter, using a papillation assay (30), we screened the KEIO *E. coli* single gene knockout library to discover genes that contribute to or prevent 5-azacytidine-induced

mutagenesis. We discovered that, predictably, uridine/cytidine kinase was essential for 5AZ cytotoxicity and mutagenic potential. However, we also discovered a dCTP deaminase that contributes to 5AZ-mediated mutagenesis. We also determined that the two primary exoribonucleases involved in RNA degradation enhance 5AZ's mutagenic effect, likely through replenishing the pools of 5AZ in the cell to be incorporated into DNA through the turnover of ribonucleosides.

Materials and Methods

***E. coli* strains –** The Keio collection is as described in Baba *et al*. (31), made from the starting strain BW25113 (32). This strain (*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$) is the starting strain used in the experiments reported here, unless otherwise stated. Briefly, each of the 3,985 strains in the KEIO collection carries a complete deletion of a different gene, with a *kan* insert replacing each gene. The base substitution tester strain, CC103 F' cam, has been described previously (21). It carries a base substitution mutation in *lacZ* (encoding β-galactosidase) on the F' plasmid, which reverts from Lac^{-} to Lac^{+} only by restoring the glutamic acid codon through a reversion by a specific base substitution (C:G→G:C). Additionally, these strains carry a mini*Tn*10*cat* insert (33) conferring chloramphenicol resistance for selection purposes (C. Tamae and J. H. Miller, unpublished).

***E. coli* genetic methods** – Unless otherwise stated, all genetic methods are as described by Miller (33).

Validation controls – A collection of close to 4,000 strains will contain some errors, and some impure strains. The latter problem can be minimized by repurifying and retesting, as was done

here. Mori and coworkers have subjected the KEIO collection to an intensive analysis aimed at uncovering errors in the collection that might arise from duplications of the target gene. They have generated a list of 14 mutants that are incorrect, and another 9 that might be incorrect (34). Ultimately, the most prudent use of such a large collection is to verify any mutants that are particularly important to the final results by PCR analysis, and/or sequencing, as we have done in a number of cases (35, 36). Here using PCR and sequencing we have verified the strain with a deletion of the *udk* and *pnp* genes. Using a primer within the *kan* gene and a primer outside the gene it replaced, we could show that a *kan* insert was at the correct position. Using internal primers for the *udk* and *pnp* genes, we could show that there was no other copy of the *udk* and *pnp* genes elsewhere in the chromosome. Controls with the starting wild-type strain showed that the internal primers were efficient.

Determination of MIC – Minimum inhibitory concentrations (37) were determined by adding ~10^3 exponentially growing cells into 2 ml LB with multiple concentrations of the desired drug, and examining the optical density after 18 hour incubation at 37°C.

Screening the *E. coli* knockout collection for mutator/antimutator strains – The Duetz cryoreplicator (38) contains 96 prongs on individual springs, allowing its frequent application to frozen glycerol cultures. The KEIO collection (31) is maintained on forty-five 96-well microtiter plates and stored at -80°C in glycerol. Material from frozen microtiter plates was transferred to microtiter plates with 0.5 ml of LB and were incubated overnight, and then the cryoreplicator was used to transfer a microdrop from each well to new microtiter plates with fresh LB medium containing 50 μg/ml kanamycin to prevent the growth of contaminants. (All of the strains in the KEIO collection are Kan^r). After 3 to 4 h of growth, these plates were transferred again (using cryoreplicator) to fresh microtiter plates containing the CC103 F' cam strain. The microtiter

plates were then incubated overnight at 37°C (18h) to allow transfer of the CC103 plasmid to knockout mutants. Using the cryoreplicator, the microtiter plates were then plated directly on the glucose minimal medium supplemented with 50μg/ml Kanamycin and 20μg/ml chloramphenicol (to select for knockout mutants containing the plasmid), and phenyl-b-D-galactoside (Pgal) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) as described previously (30). After a 4 day incubation at 37ºC, the mutator and antimutator candidates were identified as colonies that contained elevated or lowered levels of blue papillae (Figure 3-4). To confirm the (anti)mutator phenotypes, see determination of mutational frequencies (below).

Stock solution and cell treatment – 5-Azacytidine was prepared by dissolving in DMSO to a concentration of 1mg/ml, and then was added to LB media at the appropriate concentration. 5-Bromodeoxyuridine (5BdU) was prepared by dissolving in distilled water to a concentration of 1mg/ml. Chloramphenicol was prepared by dissolving in 100% ethanol to a concentration of 20mg/ml. Kanamycin was prepared by dissolving in distilled water to a concentration of 50mg/ml. For all drugs, stock solutions were then added to LB media at the appropriate concentrations, followed by the addition of ~5 x 10^4 cells and incubation for 18 hours at 37°C with aeration.

Construction of *dut* overexpression vector – PCR primers (5' - CAGAGAAAATCAAAAAGCAGGC - 3'; 5' – GTTTGCGGCTATGTTATGACG - 3') were used to amplify the segment of DNA including the *dut* gene and it's endogenous promoter using Taq polymerase (Invitrogen). The PCR product was then ligated onto the TOPO XL vector using the protocol from the TOPO XL cloning kit (Invitrogen). After chemical transformation into One Shot® TOP10F' cells (Invitrogen), the cloned plasmids were then isolated using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) and digested with restriction enzyme EcoRI (Invitrogen).

The QIAexpress pQE-60 vector (QIAGEN) was then digested using EcoRI (Invitrogen), disrupting the inducible T5 promoter in the pQE-60 vector. This vector was then ligated to the digested fragment containing the *dut* gene with endogenous promoter (Figure 3-2A). DNA sequencing was performed to verify the insert sequence and orientation.

Verification of *dut* overexpression – *E. coli* wild-type and *mutL* strains, containing pQE-60 vectors with or without the *dut* insert, were grown for 18 hours at 37°C with aeration. 50 x 10^8 cells (~15µl) were combined with equal volumes of 2X Novex® Tris-Glycine SDS sample buffer (Invitrogen), and heated at 95°C for 5 minutes. Samples were then loaded into the non-reducing Novex® NuPAGE® Tris-glycine SDS-PAGE gel (Invitrogen); Dut exists as a homotrimer held together by hydrogen bonds and not disulfide bonds, thus no reducing agent was needed. This gel was ran in the XCell *SureLock*™ Mini-Cell (Invitrogen), using Tris-glycine SDS sample buffer (Invitrogen) without sample reducing agent, at 125 volts for 90 minutes. The Bio-rad Precision Plus™ protein ladder was used as a standard. The acrylamide gel was then removed from the cell and placed in staining buffer (0.25g coomassie brilliant blue R250 in 100ml of destaining buffer) for 40 minutes under gentle shaking conditions. The gel was then placed in destaining buffer (45% methanol, 45% ddH_2O, 10% glacial acetic acid) overnight under gentle shaking conditions. In both wild-type and mismatch repair-deficient strains used in later experiments, we verified that the construct with the insert produces the desired 16.1 kDa band, which is absent in the respective strains containing the empty vector (Figure 3-2B).

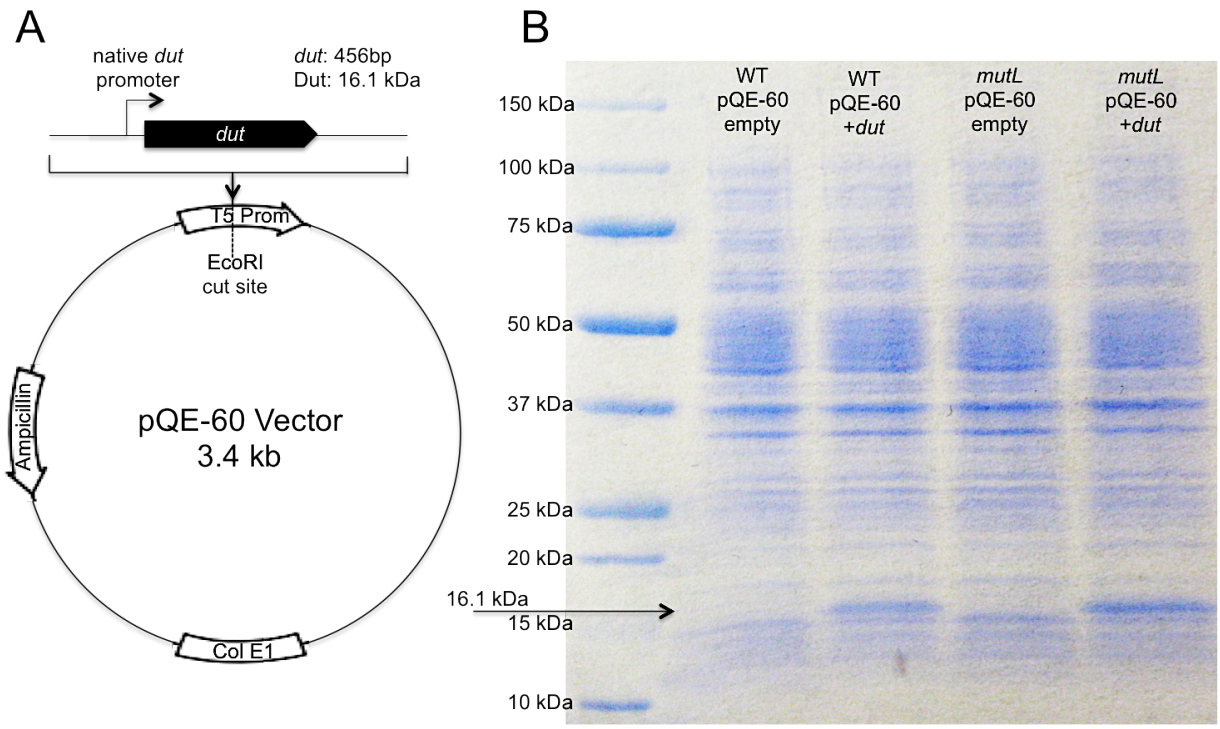


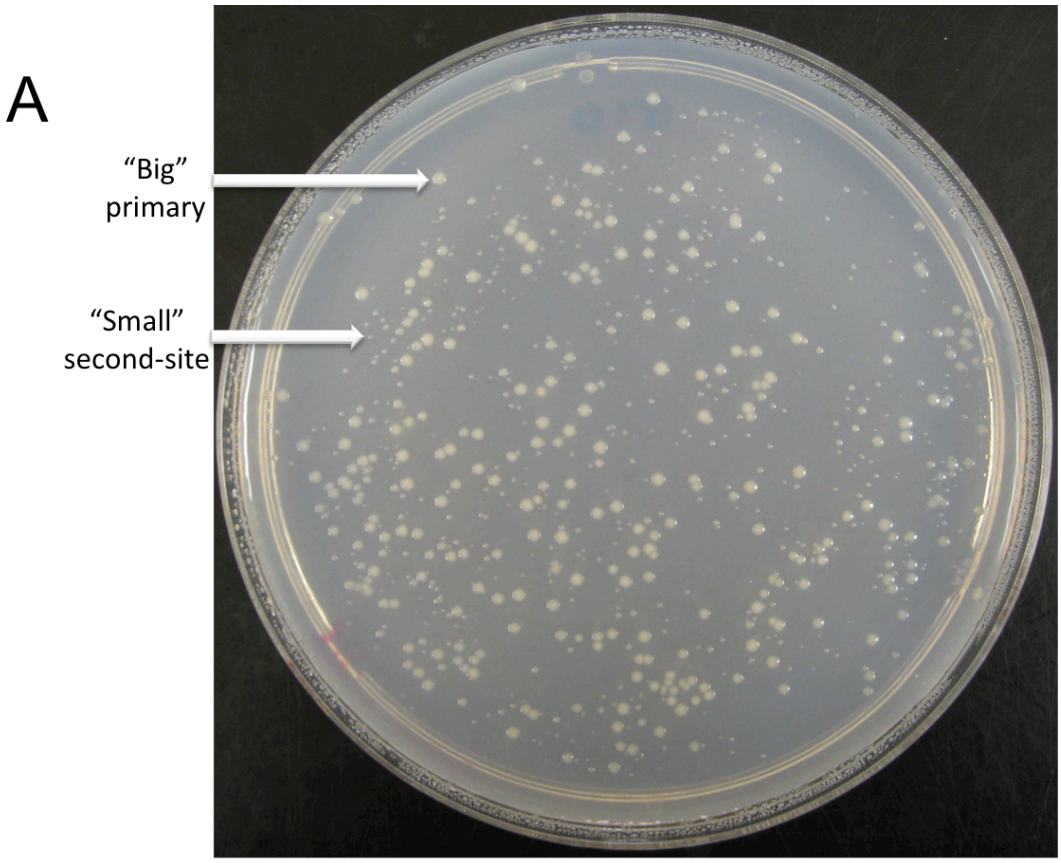
Figure 3-2. Construction and verification of the *dut* overexpression vector.

Determination of mutation frequencies – We inoculated 100-1000 cells in a series of cultures of LB or LB plus 5AZ and grown for 18 hours at 37°C with aeration, prior to plating on the appropriate medium (Lactose minimal plates, or LB plates with either 100 μg/ml rifampicin, or 20 μg/ml nalidixic acid). The mutation frequencies of Rif^r and Lac^+ revertants were determined as described previously (39). Briefly, mutation frequency (f) was determined as the median frequency from a set of cultures (the number of cultures varied from 8 to 20), and the mutation rate (μ) was determined by the formula of Drake (40). 95% confidence limits were determined according to the method of Dixon and Massey (41).

Chemicals – 5-Azacytidine, chloramphenicol, and kanamycin were purchased from Sigma (St. Louis, MO). Zebularine was a gift from Victor E. Marquez.

Results

Characterization of second-site revertants in tRNA of CC103 strain – While the primary C:G→G:C mutation in the *lacZ* gene of the CC103 F' plasmid has been previously characterized (21), lac^+ revertant colonies in mutagenesis assays (as described in 39) performed in this study revealed colonies of two different sizes (see Figure 3-3A), indicating a second mutation may be occurring. Sequencing of the large colonies reveals the primary mutation (see Material and Methods) is occurring, while sequencing of the small colonies reveals a second-site C:G→G:C mutation in the anticodon sequence of any of three (of four) genomic glutamate tRNA genes (*gltW,U,T*; Figure 3-3B). This mutated anticodon sequence then recognizes the non-reverted *lacZ* on the F' plasmid, allowing expression of active β-galactosidase. Since this mutation occurs in only one of the tRNA gene copies, this recognition by the mutated anticodon only occurs in a minority of cases, thereby resulting in a lower β-galactosidase expression and thus a slower growing (small) colony on lactose media. Because primary and second-site mutations are distinct both in genotype and phenotype, we will hereafter report lac^+ reversion frequencies separately for both "big" and "small" colonies.



B **Sequencing Summary:** *gltW*: 7/14
gltU: 4/14
gltV: 0/14
gltT: 3/14

Figure 3-3. Sequencing reveals second-site mutations in CC103 F'cam strain. A) The lac$^+$ reversion assay yields two different colony sizes, the "Big" colonies result from the primary C:G→G:C mutation on the F' plasmid, while the "Small" colonies result from a secondary mutation that occurs in the genomic glutamate tRNA genes. B) Results from sequencing 14 small colonies using primers specific for the four glutamate tRNA genes.

Screening the KEIO collection reveals two genes in the nucleotide salvage pathway responsible for 5AZ-induced mutagenesis – We screened the KEIO collection of *E. coli* single gene knockout strains for mutants that reveal either lowered or elevated mutagenesis in response to treatment with 3μg/ml 5AZ using a papillation assay (described in Materials and Methods). These assays revealed a strong decrease in mutator frequency in the absence of uridine/cytidine kinase (*udk,* Figure 3-4), dCTP deaminase (*dcd*), and a phospholytic exoribonuclease (*pnp*).

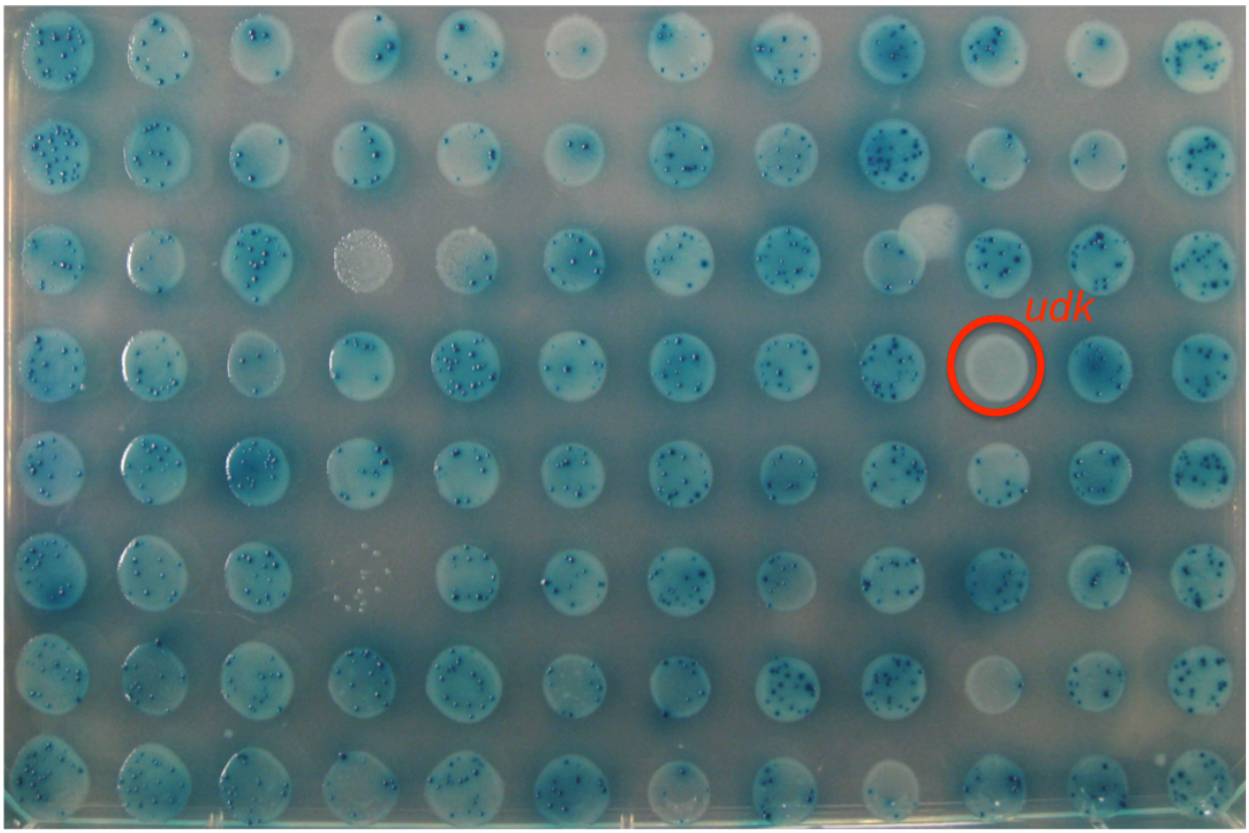


Figure 3-4. The papillation assay reveals the *udk* mutant to have decreased 5AZ-induced mutagenesis. See Materials and Methods for details on the papillation assay.

Uridine/cytidine kinase is necessary for mutagenic effect and toxicity of 5AZ – To quantify the effect on 5AZ-induced mutagenesis seen in the papillation assay above, we determined the mutation frequencies of the wild-type *E. coli* and *udk* (deficient in uridine/cytidine kinase) using a minimal lactose reversion assay and rifampicin resistance reversion assay (see Material and Methods). We did not detect any mutagenesis in *udk* strains using the minimal lactose assay, and we found a 188-fold reduction in mutagenesis using the rifampicin assay, to background levels (Figure 3-5).

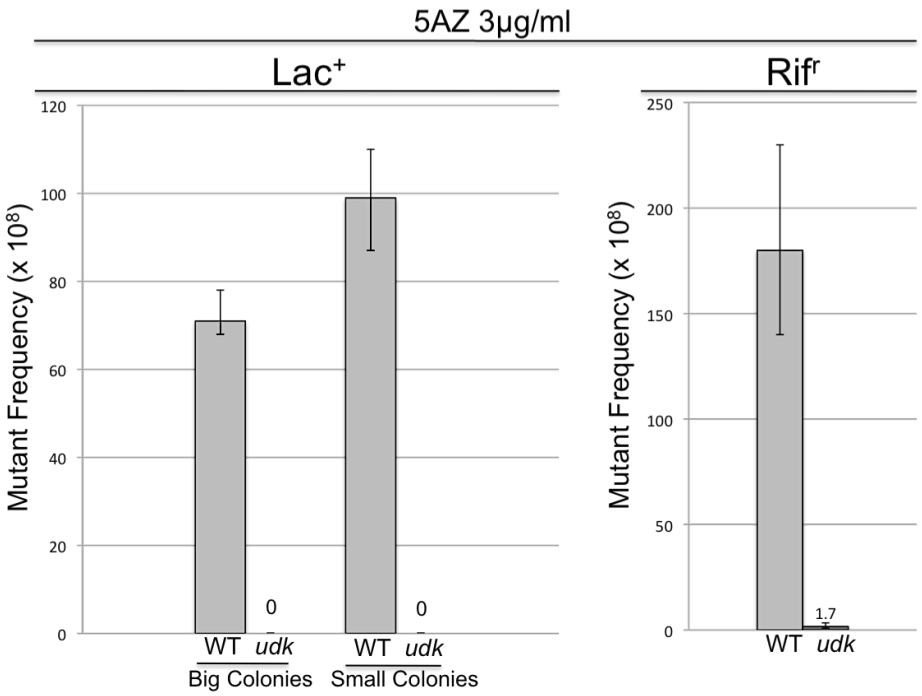


Figure 3-5. Mutagenesis in WT and UDK-deficient strains. Mutant frequencies for 5AZ-induced mutations in *lacZ*, generating lac$^+$ mutants, and *rpoB*, generating Rif^r mutants, are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details.

Because of the large effect seen, we sought to determine whether Udk was responsible for only the mutagenic effect of 5AZ, or, given its role in activating 5AZ (5, 14), if this protein was necessary for 5AZ toxicity. Thus we determined the minimum inhibitory concentration (MIC) of 5AZ in both the WT and *udk* strains. While the WT strain's MIC was between 150-200µg/ml 5AZ, the MIC of the *udk* strain is greater than 500µg/ml (Figure 3-6). This resistance along with abolishment of mutagenesis suggests that Udk is essential for 5AZ's activity.

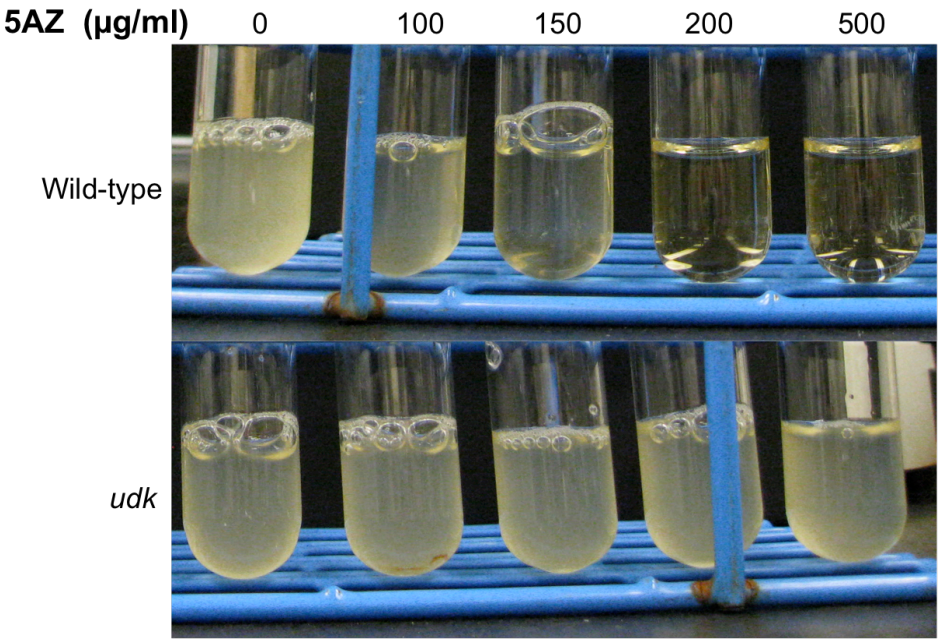


Figure 3-6. Uridine/cytidine kinase is responsible for 5AZ toxicity. *udk* mutants are insensitive to treatment with 5AZ. See Materials and Methods for determination of MIC.

Pathways producing dUTP are involved in 5AZ-mediated mutagenesis – Our papillation assay revealed 5AZ-induced mutagenesis is reduced in the dCTP deaminase mutant (*dcd*). Like with *udk*, we sought to quantify the effect on 5AZ-mediated mutagenesis in the *dcd* strain. We performed the assay to determine mutation frequency by measuring Rif^r revertants in response to 5AZ treatment (see Materials and Methods), and we observed a 10-fold reduction in mutation frequency (Figure 3-7A, left panel). Since *dcd* grows more slowly than wild-type, we tested the mutation frequency in response to another base analog, 5-bromodeoxyuridine (5BdU) to verify that the decrease seen in response to 5AZ treatment was not an artifact. Surprisingly, we discovered *dcd* has a strong mutator effect in response to 5BdU, showing an 8-fold increase in mutation frequency (Figure 3-7A, right panel). To further explore this effect, we tested the 5AZ-induced mutation frequency in a dUTPase (*dut*) overexpression strain, since this enzyme functions directly downstream of Dcd (Figure 3-7B). We discovered a 10-fold decrease in 5AZ-induced mutagenesis in this overproducer strain (Figure 3-7A, center panel).

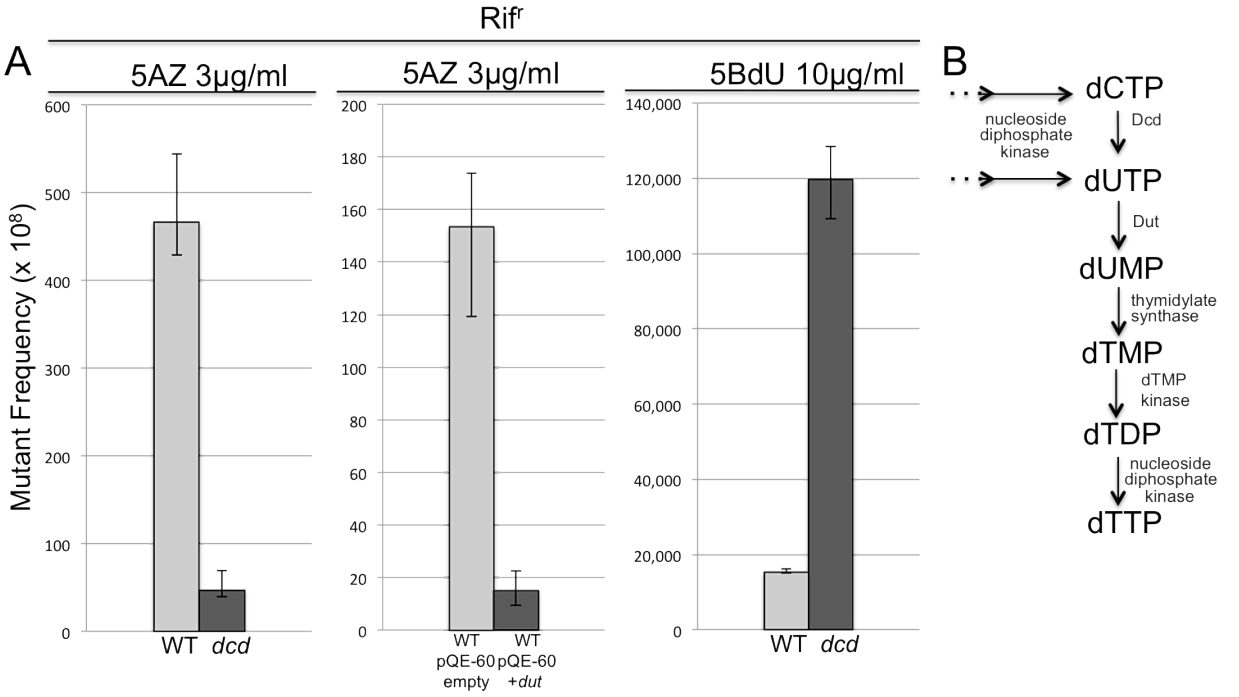


Figure 3-7. Mutagenesis in WT, DUT-overespressing, and DCD-deficient strains. A) Mutant frequencies for 5AZ- and 5BdU-induced mutations in *rpoB*, generating Rif^r mutants, are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details. B) Pathway for dTTP synthesis involving DCD and DUT.

To further explore 5AZ's involvement in the pyrimidine salvage pathway, we also tested a variety of mutants involved in pyrimidine biosynthesis/salvage, including a nucleoside diphosphate kinase (*ndk*; Figure 3-7B), uracil phosphoribosyltransferase (*upp*), uridine phosphorylase (*udp*), cytosine deaminase (*codA*), cytidine deaminase (*cdd*), (d)CMP kinase (*cmk*), polyphosphate kinase (*ppk*), thymidine/deoxyuridine kinase (*tdk*), dCMP phosphohydrolase (*yfbR*), and nucleoside transporters (*nupC* and *nupG*). None of these mutants showed a significant change in 5AZ-induced mutagenesis using either the rifampicin or lactose reversion assays (data not shown).

mRNA turnover contributes to 5AZ mutagenesis – Results from our papillation assay screen unveiled a phospholytic exoribonuclease (*pnp*) whose absence demonstrated a

decreased level of papillation. We retested this mutant's 5AZ-induced mutation frequency using the lactose reversion assay, and we saw an 8-fold reduction in big colony revertants, and a 15-fold reduction in small colony revertants (Figure 3-8, left panel). To verify that PNP's exoribonuclease activity, and not a different unidentified activity, was causing this reduction in 5AZ-mediated mutagenesis, we measured the mutation frequency in the second-most processive exoribonuclease (*rnb*). We measured a 3-fold reduction in big colony revertants, and a 1.9-fold reduction in small colony revertants (Figure 3-8, right panel). These findings implicate RNA turnover in the mutagenesis induced by 5AZ.

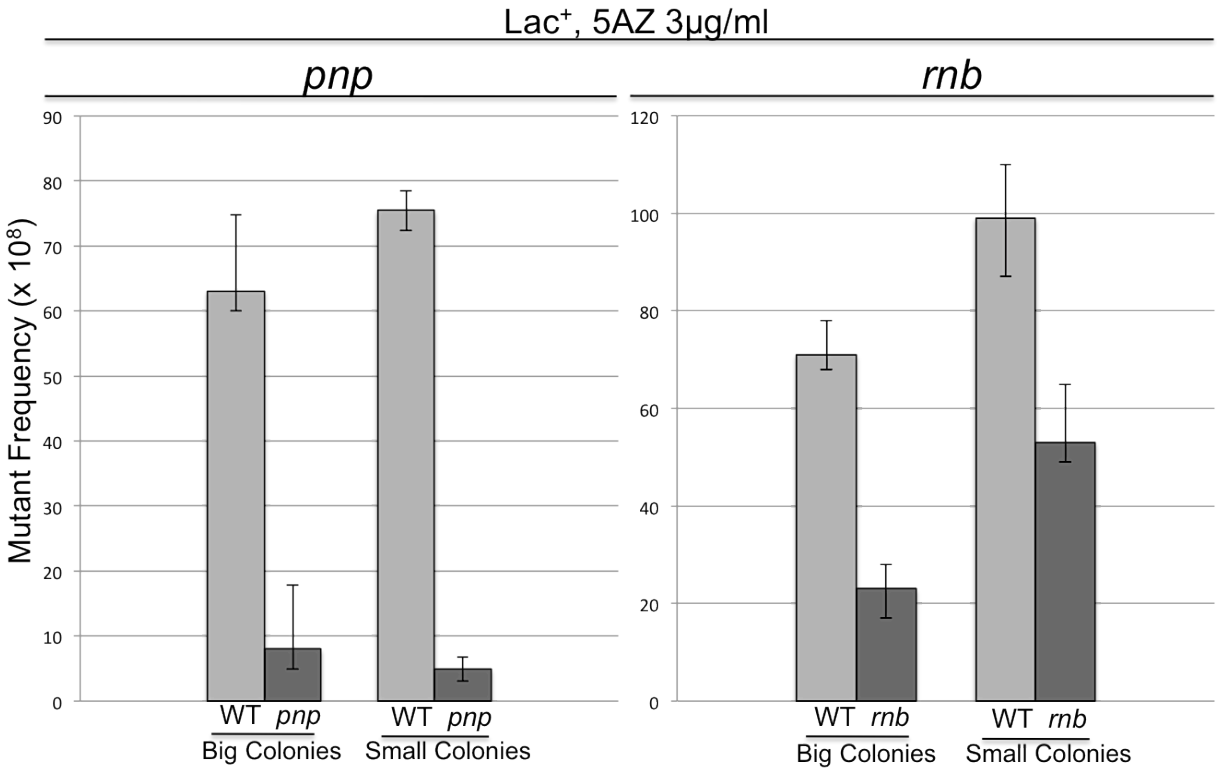


Figure 3-8. Mutagenesis in WT, PNP-deficient, and RNB-deficient strains. Mutant frequencies for 5AZ-induced mutations in *lacZ*, generating lac^+ mutants, are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details.

Discussion

In this chapter we utilized a papillation assay and an *E. coli* gene knockout library to discover proteins involved in 5AZ-induced mutagenesis. We discovered that uridine/cytidine kinase, dCTP deaminase, and exoribonucleases contribute to the occurrence of this mutagenesis, since their respective genes (*udk, dcd, pnp,* and *rnb*) showed lower levels of papillation and lower mutation frequencies in quantitative assays with respect to the wild-type strain.

It has been previously established that uridine/cytidine kinase phosphorylates 5AZ into the active nucleoside monophosphate (5, 20, 42, 43). Studies have demonstrated that bacteria and animal cells that are resistant to 5AZ show decreased activity of uridine/cytidine kinase (44-46), and 5AZ-resistant tumor samples have also shown this same decreased activity (42, 47-49). However, it has not been previously demonstrated that cells deficient in Udk have no detectable mutagenesis in response to 5AZ treatment. Our results (Figure 3-5) enforce the idea that there are no redundant functions for primary 5AZ phosphorylation in *E. coli*. Studies on 5AZ-resistant tumor cells have demonstrated that these cells also have cross-resistance with 5-aza-2-deoxycytidine, resulting from decreased activity in both proteins (44, 46). It is believed that these data suggest 5AZ is phosphorylated by both uridine/cytidine kinase and deoxycytidine kinase (42). Our findings showing the complete abolishment of 5AZ-induced mutagenesis in a *udk* strain demonstrate that there is no redundant activity for initial phosphorylation in *E. coli*. However, there is no functional counterpart to deoxycytidine kinase in *E. coli*, but Plagemann *et al.* demonstrated complete resistance to 5AZ in uridine/cytidine kinase-deficient Novikoff and P388 cells, further emphasizing the requirement of Udk for 5AZ activity. Inversely, Karlsson and coworkers demonstrated that the two human uridine/cytidine kinases do not phosphorylate deoxypyrimidines, but phosphorylate a range of pyrimidine analogs, including 6-azauridine, 5-fluorouridine, 4-thiouridine, 5-bromouridine, N^4-acetylcytidine,

N^4-benzoylcytidine, 5-fluorocytidine, 2-thiocytidine, 5-methylcytidine, and N^4–anisoylcytidine (43). Thus Udk is very important for the pharmacological activation of pyrimidine base analogs, particularly clinically relevant analogs like 5AZ and 5-fluorouracil. Interestingly, studies have demonstrated that uridine/cytidine kinase activity may increase in tumor cells lines and in response to 5AZ treatment (44, 45, 49-52)

Our findings also demonstrated that 5AZ-induced mutagenesis is lowered in both the *dcd* knockout and *dut* overexpression strains (Figure 3-7A). Dcd activity results in a depletion in the pool of cellular dCTP concentrations and has been shown to play a pivotal role in controlling relative dCTP and dTTP levels (53), and a mutant defective in *dcd* has a 5- to 10-fold elevated pool of dCTP and a 2-fold reduced pool of dTTP (54). Dcd also produces most of the dUTP in *E. coli* and *Salmonella typhimurium* for the synthesis of thymidine (Figure 3-7B, (55)). dUTPase (Dut) in turn utilizes dUTP to produce the cell's major source of dUMP for dTTP synthesis (Figure 3-7B, (56)). Cells with *dut* mutants that have lowered Dut activity show increased uracil incorporation into DNA (56). Given this pathway, and our results showing a decrease in 5AZ-mediated mutagenesis in both the *dcd* knockout and *dut* overexpression strains (Figure 3-7A), these factors suggest that increasing the pool of a uracil intermediate contributes to this mutagenesis. Since 5-azauracil can be incorporated into DNA (57), conversion of 5AZ-dCTP to 5AZ-dUTP could produce a mutagenic intermediate that is incorporated into DNA. However, since 5AZ induces C:G→G:C mispairs (26), any aza-pyrimidine analog would have to mispair with C to induce this transversion, so this mechanism is unlikely. Since *dcd* is a mutator in response to 5BdU, and 5BdU competes with uracil and thymine pools for incorporation into DNA (58), the absence of Dcd would lower the pool of dUTPs to compete with 5BdU-TP. Collectively, this information thus implicates an increase in the dUTP pool contributing to 5AZ-mediated mutagenesis, although this mechanism is unclear.

Interestingly, we did not find any significant effect in 5AZ-mediated mutagenesis when we knocked out either of the two nucleoside transporters (*nupC* and *nupG*) that transport

pyrimidines into *E. coli*. Gallagher and coworkers have demonstrated that expression of NupC restored 5AZ sensitivity in a strain deficient in *nupG* and *nupC* (59). It is likely that the redundancy of these transporters would avoid an effect in either single knockout, but previous studies have shown expression changes were sufficient enough to show an effect on toxicity. One study demonstrated that resistance to 5AZ has been correlated with impairment of uridine/cytidine uptake in the leukemic mouse models (60). Additionally, *in vitro* analysis of patient cells showed a correlation between sensitivity to treatment with nucleoside analog chemotherapeutics and expression of nucleoside transporters (61-63), including 5-aza-2'-deoxycytidine (64). This correlation was further demonstrated with 5AZ using an ENT-1 transporter inhibitor, nitrobenzyl-mercaptopurine (65). However, there are two putative pyrimidine transporters, *nupX* and *rutG*, that could possibly be involved in 5AZ import (66, 67). However, given that these genes did not show a change in papillation when we screened the KEIO collection, it is likely that together these importers perform redundant functions of 5AZ import.

The decrease in mutagenesis in response to 5AZ treatment seen in the *pnp* and *rnb* mutants (Figure 3-8) implicates RNA turnover in contributing to 5AZ-mediated mutagenesis. These exoribonucleases are the two most processive in RNA degradation. Rnb is a hydrolytic enzyme, which generates a pool of rNMPs and is less processive than Pnp (68). Pnp is a phospholytic exonuclease and generates a pool of rNDPs (69, 70). These pools then mix with the pools from *de novo* biosynthesis (71, 72). Thus, these proteins would recycle cellular 5AZ pools originally incorporated in RNA and produce renewed pools of 5AZ-MP and 5AZ-DP. Because ribonucleosides are converted to deoxyribonucleosides by ribonucleoside diphosphate reductase (RNR) at the diphosphate level, the 5AZ-DP's generated by PNP-mediated degradation of RNA can be easily shunted into DNA to result in further mutagenesis. 5AZ-MP products from Rnb processing require an additional phosphorylation step to be converted by RNR. The slow conversion of UMP/CMP to UDP/CDP, respectively, in cells is a rate-limiting

step in *de novo* pyrimidine biosynthesis (69), so 5AZ-MP conversion to 5AZ-DP must go through this bottleneck. Thus, combined with the lower processivity of Rnb, it is logical that while *rnb* decreases 5AZ-induced mutagenesis, it does so at a level lower than that of *pnp*. However, since there is 10-fold more RNA in the in the cell than DNA (73), this RNA turnover likely exacerbates mutagenesis by 5AZ by recycling the analog in the cell.

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CHAPTER 4: The Origin of Mutations in the Pools of Nucleoside Diphosphates Generated by the Degradation of RNA

Summary

Polynucleotide phosphorylase (PNP) plays a central role in RNA degradation, generating a pool of rNDP's that can be converted to dNDPs by ribonucleotide reductase. We show here that the rNDP pool generated by polynucleotide phosphorylase (PNP) degradation of RNA is responsible for spontaneous mutations that result from replication errors, and these are normally repaired by the mismatch repair (MMR) system, since these mutations are not observed in a PNP-deficient strain. This is true for base substitution mutations that occur in either the *rpoB* gene leading to Rif^r, in the *gyrB* gene leading to Nal^r, and for both base substitutions and frameshift mutations that occur in the *lacZ* gene. The PNP-derived pool is also ultimately responsible for the observed mutations in the *mutT* mutator background, and after treatment with 5-bromodeoxyuridine. However, mutation frequencies are not reduced in a *mutY* mutator background, or after treatment with 2-aminopurine. These results argue that the rNDP pools derived from RNA degradation fuel replication to the point that replication errors escape the exonucleolytic editing function of DNA polymerase, but can be dealt with by the MMR system. In PNP-deficient strains, the reduced rNDP pools limit replication to the point where the editing function can correct replication errors, and the MMR system is not required.

Introduction

Elucidating the pathways that lead to mutations resulting from replication errors, arising either spontaneously or induced by chemical agents, has intrigued molecular biologists ever since the elucidation of the structure of DNA allowed one to pose this question in molecular

terms (1, 2). What is the actual source of these mutations? Different tautomeric forms of the four bases in DNA or their analogs have been considered as provoking errors directly (2-6), or even indirectly, for instance by affecting the pools of nucleoside triphosphates (5-7). Also, the field has defined a myriad of repair systems aimed at preventing or repairing DNA damage (4), and also aimed at correcting errors of DNA replication (8, 9). In humans, defects in one of a number of repair systems leads to inherited cancer susceptibilities (*e.g.* (10, 11)). In *Escherichia coli*, the replicating DNA polymerase (Pol III) contains an editing subunit that corrects numerous replication errors (12-14). Directly following replication, the mismatch repair system recognizes still uncorrected mismatches and repairs them, using the pattern of methylation to distinguish the template strand form the newly synthesized strand (8, 9). Mutants lacking any one of the components of this system (*e.g.* MutH, MutL, MutS, UvrD) have sharply elevated mutation rates that involve transitions (A:T→G:C or G:C→A:T; (15-17)), or short indels (insertion/deletions) at repeated sequences, such as monotonous runs of G's, or A's on the same strand (17, 18). The size and balance of the nucleoside triphosphate (NTP) pools are important for replication fidelity (19). Not only do unbalanced pools provoke an increase in mutagenesis (19-21), but an increase in the pools of all four dNTPs also leads to increased mutations (22, 23).

One widely used approach to studying mutational pathways is to find mutants with increased mutation rates, or "mutators" (*e.g.* (24, 25)). A more difficult approach is to detect mutants with lowered mutation rates, or "antimutators." In a study to be reported elsewhere (E. Becket and J. H. Miller, unpublished), we screened the *E. coli* KEIO collection of gene knockouts for mutants with a reduced rate of mutagenesis induced by the base analog 5-azacytidine (5AZ). We found that mutants deleted for the *pnp* gene, encoding polynucleotide phosphorylase (PNP), had lower levels of 5AZ-induced mutagenesis. Here, we report the effects of PNP-deficiency on mutagenesis induced by two other base analogs, 5-bromodeoxyuridine (5BdU), and 2-aminopurine (2AP), as well as on spontaneous mutations

that result from replication errors. These studies reveal that the pool of nucleoside diphosphates generated by PNP-mediated degradation of RNA drives spontaneous and certain mutagen-induced mutations.

Materials and Methods

***E. coli* strains** – The Keio collection is as described in Baba *et al*. (26), made from the starting strain BW25113 (27). This strain (*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$) is the starting strain used in the experiments reported here, unless otherwise stated. Briefly, each of the 3,985 strains in the KEIO collection carries a complete deletion of a different gene, with a *kan* insert replacing each gene. The base substitution tester strains, CC102, and CC106, have been described previously (15). They carry a base substitution mutation in *lacZ* on the F' plasmid, which reverts from Lac^{-} to Lac^{+} only by restoring the glutamic acid codon through a reversion by a specific base substitution for each (G:C→A:T, G:C→C:G, and A:T→G:C respectively). Additionally, these strains carry a mini*Tn*10*cat* insert (28) conferring chloramphenicol resistance for selection purposes (C. Tamae and J. H. Miller, unpublished). Also, *mutL*, *mutT*, and *mutY* derivatives of BW25113 were prepared by P1 transduction from strains carrying miniTn10tet inserts (C. Tamae and J. H. Miller, unpublished) in either *mutL*, *mutT* or *mutY*.

***E. coli* genetic methods –** Unless otherwise stated, all genetic methods are as described by Miller (28).

Validation controls – A collection of close to 4,000 strains will contain some errors, and some impure strains. The latter problem can be minimized by repurifying and retesting, as was done

here. Mori and coworkers have subjected the KEIO collection to an intensive analysis aimed at uncovering errors in the collection that might arise from duplications of the target gene. They have generated a list of 14 mutants that are incorrect, and another 9 that might be incorrect (29). Ultimately, the most prudent use of such a large collection is to verify any mutants that are particularly important to the final results by PCR analysis, and/or sequencing, as we have done in a number of cases (30, 31). Here using PCR and sequencing we have verified the strain with a deletion of the *pnp* gene. Using a primer within the *kan* gene and a primer outside the gene it replaced, we could show that a *kan* insert was at the correct position. Using internal primers for the *pnp* gene, we could show that there was no other copy of the *pnp* gene elsewhere in the chromosome. Controls with the starting wild-type strain showed that the internal primers were efficient.

We transduced the *pnp* deletion/*kan* insertion carrying strain used in these experiments with a linked *Tn*10 element (from strain CAG12153; (32)). A majority of the Tet^r transductants had crossed out the *pnp* deletion/replacement, as determined by the loss of Kan^r, which was accompanied by the restoration of the normal colony size. We tested two of these transductants for the mutation frequency (Rif^r) in the presence of 5BdU and found that they had restored the high mutation frequency characteristic of PNP-proficient strains (data not shown).

The major hotpsot in *rpoB* that occurs after 5BdU treatment or in MMR-deficient strains represents two thirds of the mutations detected (see Figure 4-3, and (33)). To verify that cells with this mutation still confer the Rif^r phenotype in a PNP-deficient strain, we carried out a reconstruction experiment. We transduced this mutation from the wild-type into a *pnp* derivative without selecting for Rif^r in two steps, by using a linked *Tn*10 in the *argE* gene (from strain CAG12185; (32)). First, the *argE::Tn*10 was transduced into the *pnp* deletion/*kan* replacement strain, scoring for retention of the Kan^r phenotype. Then the strain was transduced to Arg^+ using a lysate prepared on the Rif^r strain carrying the *rpoB* allele in question. Arg^+

transductants were scored for retention of the pnp/kan deletion replacement, and also for Rif^r. The expected 60% of the Arg^+ strains formed Rif^r colonies (in the *pnp* background).

Stock solution and cell treatment – 5-bromodeoxyuridine was prepared by dissolving in distilled water to a concentration of 2mg/ml. Solid 2-aminopurine was added directly to LB media for a concentration of 700μg/ml, which was then distributed in 3 ml aliquots, followed by the addition of ~5 x 10^4 cells and incubation for 24 hours at 37°C with aeration.

Determination of mutation frequencies – We inoculated 100-1000 cells in a series of cultures of LB or LB plus 2AP or 5BdU and grown for 18 hours at 37°C with aeration, prior to plating on the appropriate medium (Lactose minimal plates, or LB plates with either 100 μg/ml rifampicin, or 20 μg/ml nalidixic acid. The mutation frequencies of Rif^r and Lac^+ revertants were determined as described previously (21). Briefly, mutation frequency (f) was determined as the median frequency from a set of cultures (the number of cultures varied from 8 to 20), and the mutation rate (μ) was determined by the formula of Drake (34). 95% confidence limits were determined according to the method of Dixon and Massey (35).

Chemicals – 5-bromodeoxyuridine, 2-aminopurine, tetracycline, chloramphenicol, and kanamycin were purchased from Sigma (St. Louis, MO).

Results

Base analog-induced mutagenesis – Figure 4-1A shows that 5BdU mutagenesis is reduced in a *pnp* mutant on the order of 1,000-fold relative to the PNP^+ starting strain, as measured by the frequency of Rif^r mutants. However, 2AP mutagenesis is not lowered in a *pnp* mutant (Figure 4-

1B). We have verified the effect of a PNP deficiency on mutagenesis in two additional systems. Figure 4-1C shows that when monitoring mutagenesis by the frequency of nalidixic acid resistance (Nal^r), again 5BdU displays a reduction on the order of 1,000 fold in a *pnp* strain, whereas 2AP mutagenesis is not reduced (Figure 4-1D). Moreover, when the reversion of specific *lacZ* alleles is used to measure mutagenesis, 5BdU-induced mutagenesis is also sharply reduced (Table 4-1). CC102 reverts to wild-type via a specific G:C→A:T transition, and CC106 reverts via a specific A:T→G:C transition (15). Because PNP and RNase II (RNB) are the two enzymes responsible for RNA degradation and the resulting rNDP and rNMP pools (36, 37), respectively, derived from this degradation, we tested *rnb* (encoding RNB) mutants. Figure 4-2 shows that a deletion of *rnb* also results in a reduction of 5BdU-induced mutagenesis, although the modest 3.5-4.0 fold decrease is far less than that seen with *pnp* deletion strains.

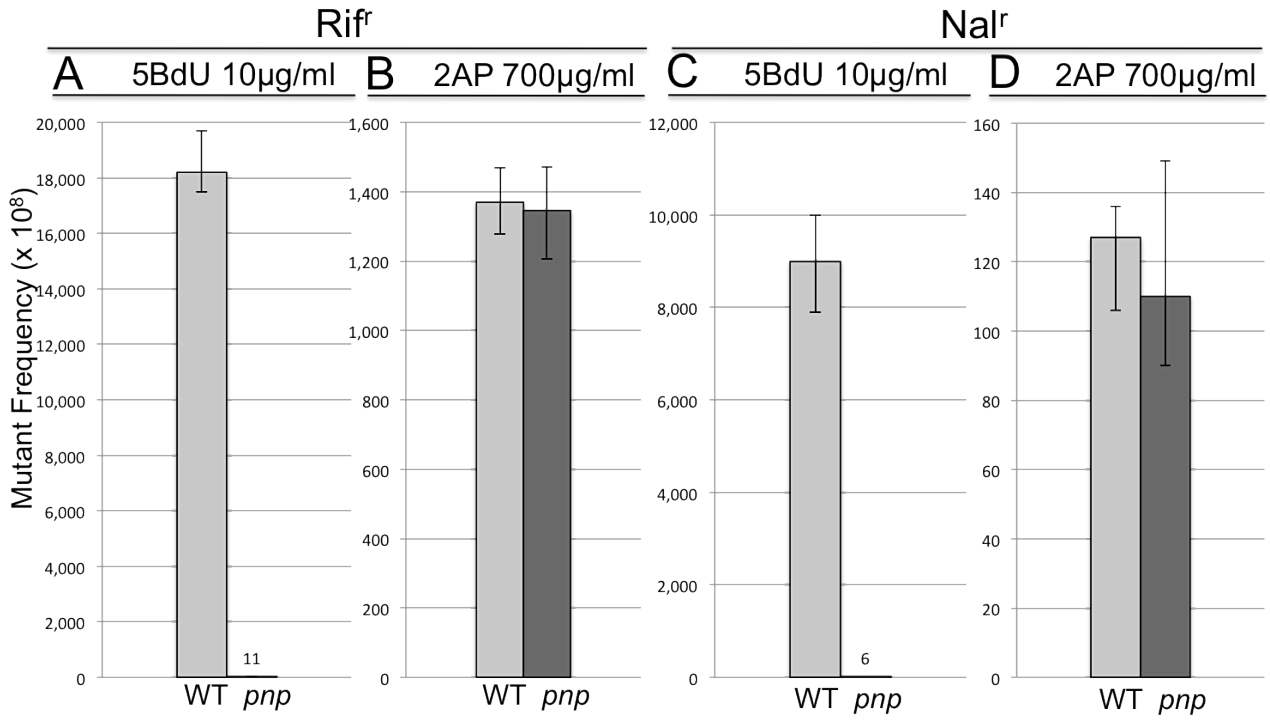

Figure 4-1. Mutagenesis in WT and PNP-deficient strains. Mutant frequencies for 5BdU and 2AP induced mutations in *rpoB*, generating Rif^r mutants, and *gyrA*, generating Nal^r strains, are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details.

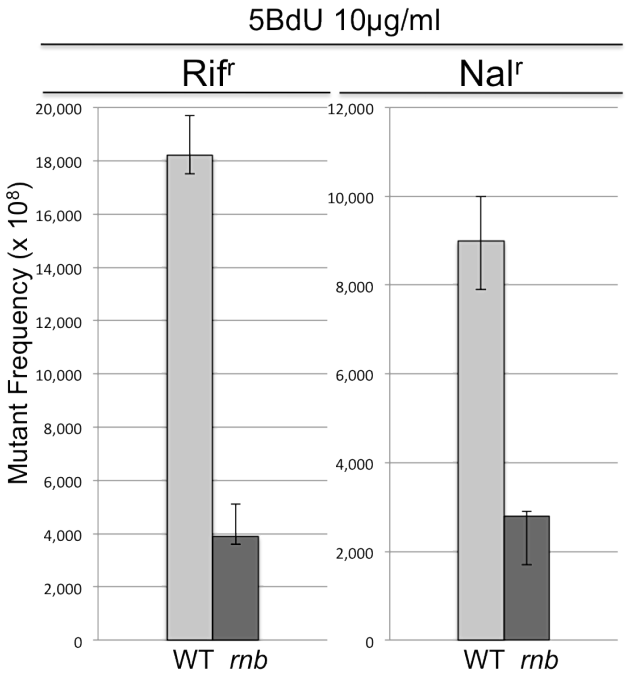

Figure 4-2. 5BdU mutagenesis in WT and RNB-deficient strains. (See legend to Figure 4-1).

Table 4-1

Frequencies (*f*) and mutation rates (μ)[a] in CC102 and CC106 after treatment with 5BdU 10µg/ml

	CC102 WT	CC102 *pnp*	CC106 WT	CC106 *pnp*
f (x10^8)	470 (420-1,300)[b]	3 (0-7.5)	760 (410-1,900)	0 (0-1.7)
μ (x10^8)	60 (54-143)	0.86 (0-1.8)	90 (53-206)	0 (0-0.57)

[a] The *rpoB* mutation frequencies (*f*) per cell are calculated by dividing the median number of mutants by the average number of cells in a series of cultures, and the mutation rates (μ) was determined in the method of Drake (1991; see Materials and Methods).
[b] Values in parentheses are 95% confidence limits.

Pathway of 5BdU-induced mutagenesis – The study of mutagenic spectra has revealed that spontaneous mutations, as well as those induced by each mutagen treatment and mutator background result in a characteristic pattern of hotspots and coldspots (*e.g.* (33, 38-40)), most probably as a result of the surrounding sequences. Analysis of the spectra of mutations in *rpoB* leading to Rif^r is a case in point (33, 41). Figure 4-3 shows the pattern of transition mutations (A:T→G:C, G:C→A:T) in *rpoB* for the base analogs 2AP (33), zebularine (ZEB; (41)), and 5BdU (this work), as well as the mutagen ultraviolet irradiation (UV; (33)), the mismatch repair

deficient mutator *mutS* (33) and the unmutagenized wild-type (33, 42). There are 29 sites detected in *rpoB* that lead to Rif^r via a transition (42) and these are listed by base pair number in *rpoB* in terms of the percentage of the total number of Rif^r mutants analyzed. The A:T→G:C mutations are on the left portion of each panel, and the G:C→A:T mutations are on the right portion of each panel. The total number of mutations analyzed in each sample is as follows: 2AP, 30; ZEB, 156; 5BdU, 194; UV, 40; *mutS* (MMR$^-$), 174; SPON, 298. The percentage of the total mutations that result from transitions is close to or over 90% for all but the SPON (WT) set. These percentages are: 2AP, 97%; ZEB, 92%; 5BdU, 99%; UV, 88%; *mutS*, 98%; SPON, 47%. What is evident from Figure 4-3 is that while each mutagen or mutator shows a different pattern of transition frequencies at the available sites, 5BdU displays a pattern that is virtually identical to that of a mismatch repair-deficient strain (*mutS*). Although one might argue that this pattern is coincidental, we see the same pattern among sites in the *gyrB* gene that lead to Nal^r. Table 4-2 displays our analysis of *gyrB* mutations leading to Nal^r. There are less sites in *gyrB* than in *rpoB*, but of the 18 sites detected, 8 involve transitions, and 4 of these represent the A:T→G:C transition that is favored by 5BdU in the *rpoB* gene (Figure 4-3). One of these sites, at position 247, is the prominent hotspot in MMR-deficient strains (*mutL*), and after 5BdU treatment. Note that 2AP does not have the same hotspot. Moreover, overproducing MutL on a plasmid reduced the level of 5BdU mutagenesis up to 150-fold (data not shown). The results with 5AZ, and for *mutT* and *mutY* are also shown here to underscore the fact that this system correctly identifies the known mutagenic specificity of these treatments or strain backgrounds. Namely, 5AZ is specific for G:C→C:G changes, *mutT* for A:T→C:G changes, and *mutY* for G:C→T:A changes (15, 41, 43, 44). Taken together, the results in Figure 4-3 and Table 4-2 strongly indicate that 5BdU is not targeting mutations directly, as other base analogs such as 2AP, ZEB, and 5-azacytidine do, but instead acts indirectly, by increasing normal DNA polymerase replication errors and saturating mismatch repair.

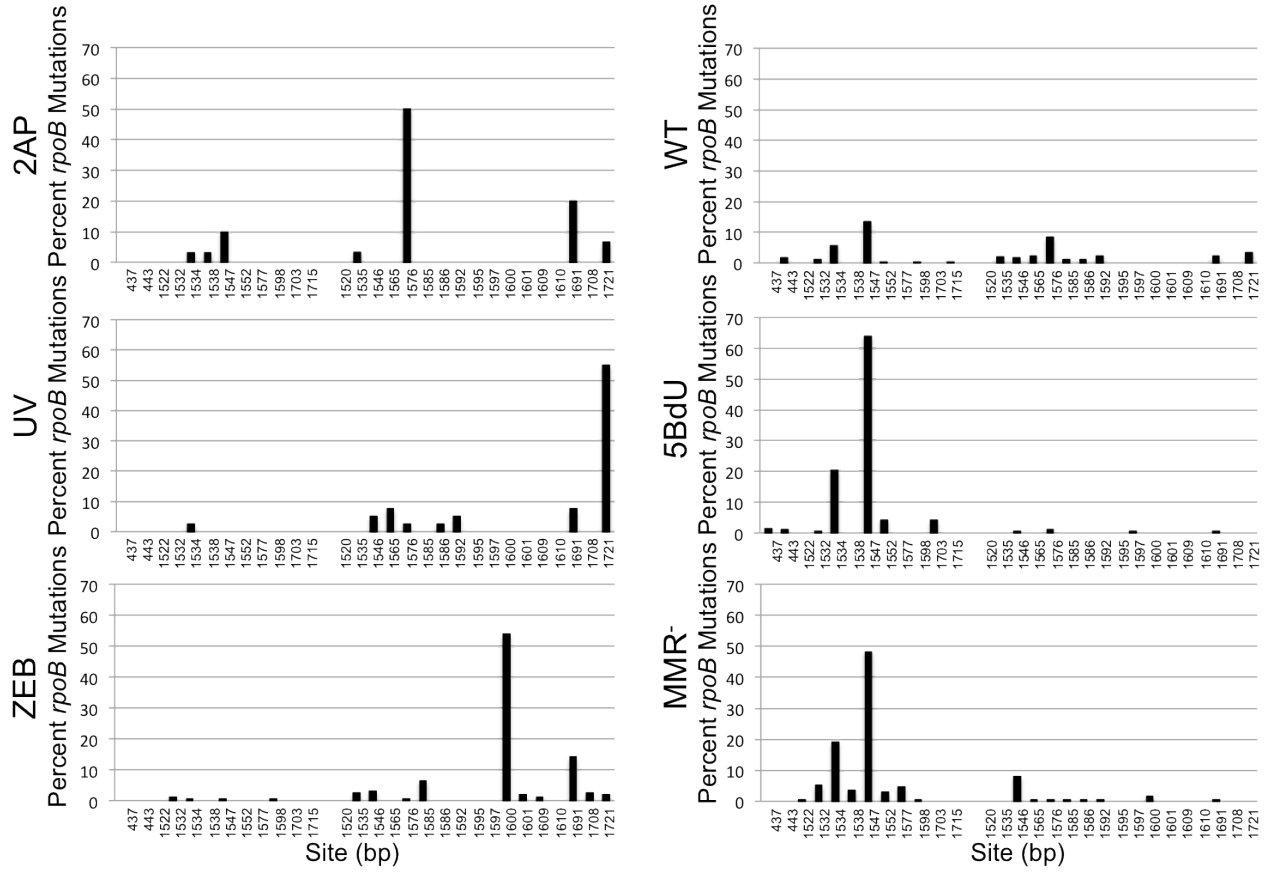

Figure 4-3. Transition mutations in *rpoB* leading to the Rif^r phenotype occurring spontaneously or after treatment with different mutagens. A:T→G:C mutations are shown in the left portion of each diagram, and G:C→A:T mutations in the right portion. The height of each bar represents the percentage of all the *rpoB* mutations detected in that sample. For further details consult the text.

Table 4-2
Distribution of mutations in *gyrA*

Site (bp)	AA Change	bp Change	WT	WT 5Bdu	WT 2AP	*mutL*	*mutT*	*mutY*	WT 5AZ
215	D → G	AT → GC	0	2	0	0	0	0	0
245	D → G	AT → GC	1	1	0	5	0	0	0
247	S → P	AT → GC	0	0	0	1	0	0	0
260	D → G	AT → GC	17	57	2	67	0	0	2
152	A → V	GC → AT	0	0	0	0	0	0	0
244	D → N	GC → AT	0	0	2	0	0	0	0
248	S → L	GC → AT	17	0	8	10	0	1	0
259	D → N	GC → AT	5	0	8	1	0	0	0
241	G → C	GC → TA	2	0	0	0	0	1	0
259	D → Y	GC → TA	11	0	0	0	0	0	0
356	A → E	GC → TA	0	0	0	0	0	6	0
245	D → A	AT → CG	0	0	0	0	0	0	0
247	S → A	AT → CG	1	0	0	0	7	0	0
260	D → A	AT → CG	1	0	0	0	19	0	0
260	D → V	AT → TA	0	0	0	0	0	0	0
248	S → W	CG → GC	2	0	0	0	0	0	31
259	D → H	CG → GC	1	0	0	0	0	0	4
316	Q → E	CG → GC	0	0	0	0	0	0	0
Total			55	60	20	84	26	8	37

Spontaneous base substitution mutations – We examined the effects of deleting *pnp* on spontaneous mutations. While the effects on the frequency of spontaneous base substitution mutations in a MMR-proficient background are minimal (Table 4-3), the effects in a MMR-deficient strain are dramatic, as foreshadowed by the results with 5BdU. Figure 4-4 shows that in three different systems the introduction of a *pnp* deletion results in a lowering of Rif^r mutations by 140-fold, Nal^r mutations by 160-fold, Lac^+ mutations resulting from an A:T→G:C transition in l*acZ* by 64-fold, and from a G:C→A:T transition 78-fold. These levels are now very near to those seen in a MMR-proficient strain (Table 4-3). To demonstrate that these reductions are not simply an artifact of the *pnp* background, we show that the increase in mutation frequency that occurs in a *mutY* background (44) is not reduced in a *mutY pnp* background (Figure 4-5A,B). However, the increased level of mutations in a *mutT* mutator background is eliminated when *pnp* is deleted (Figure 4-5C,D), as the levels are now close to those seen in a wild-type strain.

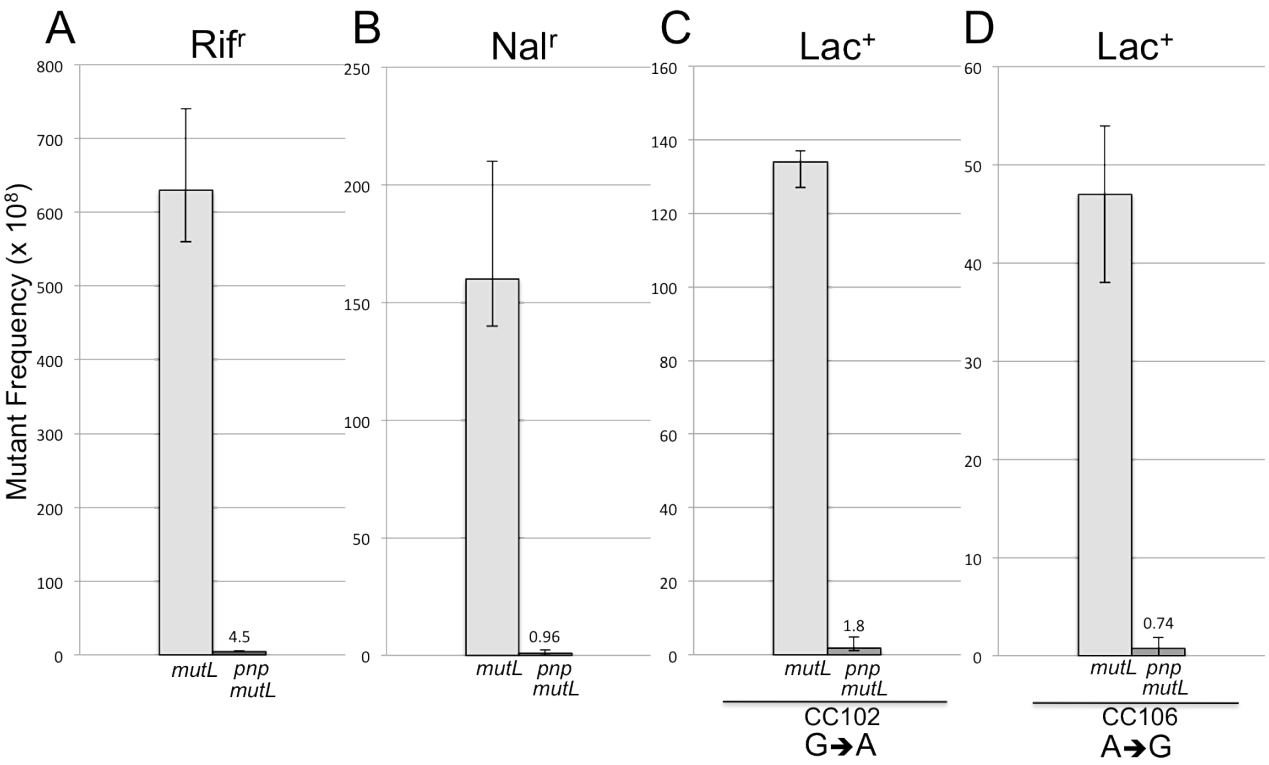

Figure 4-4. Mutant frequencies of MutL-deficient strains in otherwise WT or PNP-deficient backgrounds. The frequencies of mutants to Rif^r, Nal^r or Lac$^+$ are shown in each case, reflecting mutations in *rpoB*, *gyrA*, and *lacZ*, respectively (see legend to Figure 4-1 and text).

Table 4- 3

Spontaneous frequencies (*f*) and mutation rates (μ)[a] in wild-type and *pnp* strains

	Rif^r		Nal^r	
	Wild-Type	*pnp*	Wild-Type	*pnp*
f (x10^8)	3.7 (3.0-4.6)[b]	2.9 (2.3-3.8)	0.46 (0.23-0.69)	0.39 (0-0.79)
μ (x10^8)	0.81 (0.68-0.96)	0.68 (0.57-0.85)	0.15 (0.090-0.21)	0.14 (0-0.24)

[a] The *rpoB* mutation frequencies (*f*) per cell are calculated by dividing the median number of mutants by the average number of cells in a series of cultures, and the mutation rates (μ) was determined in the method of Drake (1991; see Materials and Methods).
[b] Values in parentheses are 95% confidence limits.

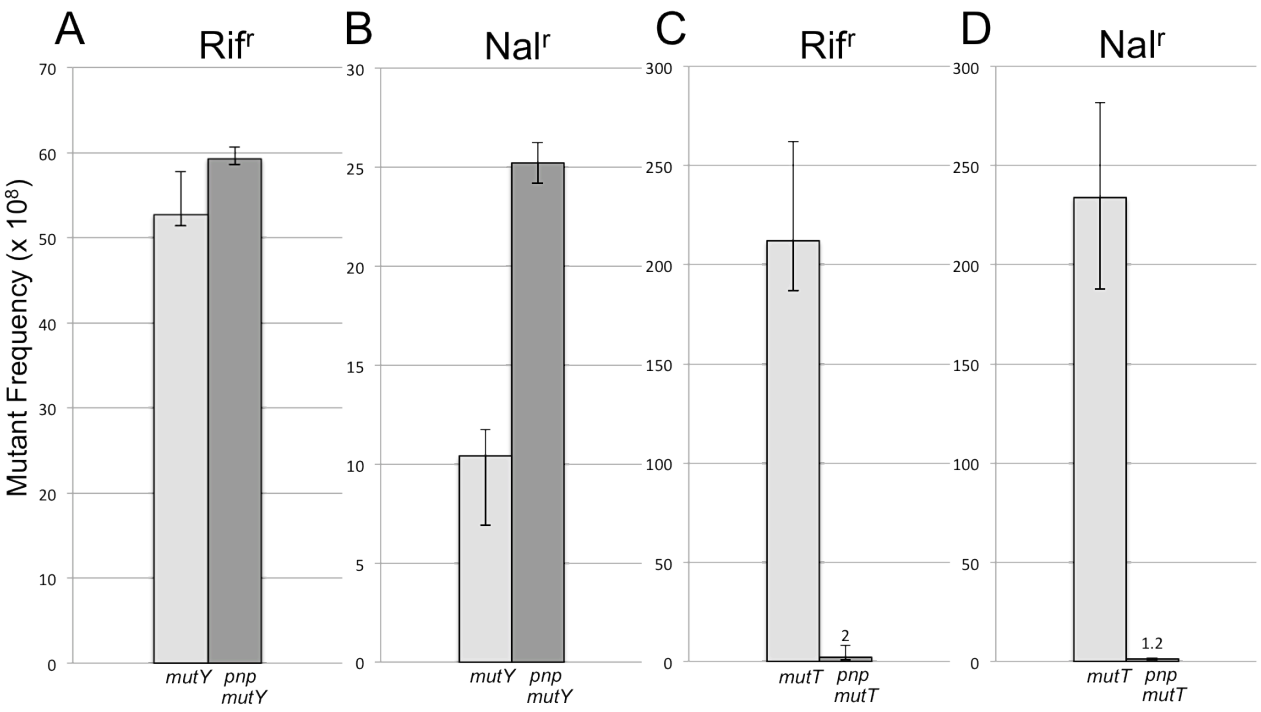

Figure 4-5. Mutant frequencies in WT and PNP strain backgrounds that are also either MutY- or MutT-deficient. (See legend to Figure 4-1 and text)

Frameshift mutations – Frameshift mutations at repeated mono- or dinucleotides are frequent replication errors that are repaired by the MMR system (8, 17, 18). Strain CC107 reverts to Lac^+ via the addition of a -G- in a monotonous run of 6 G's (18). Table 4-4 shows the frequency of this frameshift mutation in cultures of wild-type and MMR-deficient strains. 5BdU treatment also increases the level of these frameshifts to near the level of that seen in a MMR-deficient strain. Strains deleted for *pnp* reduce the levels of this mutation 330-fold in MMR-deficient strains, and in 5BdU treated cells down to even below the level of untreated wild-type strains, a several thousand-fold effect (Table 4-4).

Table 4-4

Frequencies (f) and mutation rates (μ)[a] of frameshift mutations with and without treatment with 5BdU 10µg/ml

				5BdU 10 µg/ml	
	CC107 WT	CC107 *mutL*	CC107 *pnp mutL*	CC107 WT	CC107 *pnp*
f (x10^8)	56 (45-79)[b]	76,000 (69,000-78,000)	230 (180-360)	27,000 (23,000-34,000)	7 (4.5-8.8)
μ (x10^8)	8.9 (7.4-12)	5,900 (5,300-6,000)	30 (24-45)	2,200 (1,900-2,700)	1.5 (1.0-1.8)

[a] The *rpoB* mutation frequencies (f) per cell are calculated by dividing the median number of mutants by the average number of cells in a series of cultures, and the mutation rates (μ) was determined in the method of Drake (1991; see Materials and Methods).
[b] Values in parentheses are 95% confidence limits.

Discussion

We show here that deleting the gene (*pnp*) encoding polynucleotide phosphorylase (PNP) virtually eliminates the dramatic increase of spontaneous mutations that occurs when the mismatch repair system (MMR) is inactivated (*e.g.* in *mutS* or *mutL* strains). This is true for base substitution mutations (Figure 4-4) that occur in either the *rpoB* gene leading to Rif^r, in the *gyrB* gene leading to Nal^r, and for both base substitutions and frameshift mutations that occur in the *lacZ* gene (Tables 4-1 and 4). This is also true for base substitutions resulting from inactivation of the *mutT* gene (Figure 4-5) that sanitizes the pool of oxidized dGTP's (45) and rGTPs (46) by converting them to monophosphates. Mutations that are elevated in a MutY-deficient background, however, are not decreased (Figure 4-5), as the mutations result from oxidation of guanines in the DNA itself. Moreover, there is a similar striking reduction in mutations generated after continuous growth in 5BDU (Figure 4-1, Table 4-4). Although 5BdU is incorporated into DNA, it has been suggested that it may cause mutations indirectly, rather than directly targeting them (5-7). An inspection of the mutational spectra in *rpoB* argues that 5BdU induces mutations indirectly by enhancing normal replication errors and saturating mismatch repair (Figure 4-3; see text). In contrast, 2AP-induced mutations are not lowered in a PNP-deficient strain (Figure 4-1).

How can the absence of PNP virtually eliminate spontaneous mutagenesis under certain conditions? PNP plays a central role in RNA degradation, generating a pool of rNDP's (36, 37)

that mix with the pools from *de novo* biosynthesis (Figure 4-6; (14, 47)). One should note that *de novo* pyrimidine biosynthesis is carried out at a low level, the rate-limiting step being the conversion of UMP/CMP to UDP/CDP, respectively (36). The primary source of pyrimidines for DNA synthesis may in fact be the pool of nucleosides derived from RNA. There is 10-fold more RNA in the in the cell than DNA (48), and that ribonucleoside pools have been measured to greatly exceed deoxyribonucleoside pools. For instance, in yeast, the four rNTPs are 36-190 more prevalent than the four dNTPs (49). Also, because ribonucleosides are converted to deoxyribonucleosides by ribonucleoside diphosphate reductase (RNR) at the diphosphate level, the rNDP's generated by PNP-mediated degradation of RNA can be easily shunted into DNA (Figure 4-6). We can therefore envision several explanations for the effect on mutagenesis of deleting *pnp*. One possibility is that the PNP-generated pools normally result in the incorporation of ribonucleosides into DNA, and that this has mutagenic consequences. Direct measurement of ribonucleosides incorporated into DNA by yeast DNA polymerases *in vitro* indicates that rNMP is incorporated between once for every 625 dNMPs to once per 5,000 dNMPs, depending on the polymerase (49). These are removed in vivo by a process involving RNase H2, and mutants lacking RNase H2 in yeast have increased levels of levels of spontaneous mutations that involve primarily short deletions or insertions of 2-5 bp (50-52). Models for the generation of these mutations involve processing by topoisomerase I, and misalignment (53). Interestingly, these mutations are not increased in a MMR-deficient strain (51). However, this mechanism would not account for most of the mutations that we score in the work reported here, as these are base substitution mutations that are subject to mismatch repair. Moreover, in the *E. coli* strain background we are using, RNase H1 or RNase H2 deficient strains (*rnhA* or *rnhB*) are not mutators for the *rpoB* system used here, in either an otherwise wild-type background or a MMR-deficient background (data not shown).

A second and related possibility is that degradation of RNA by PNP provides uracil in the form of rUDP. This is a required intermediate in the incorporation of dTTP into DNA.. The

rUDP is converted to dUDP and then dUTP, and then dUTPase converts dUTP to dUMP, from which it is converted into dTMP. dUTPase action prevents dUTP from being incorporated into DNA. Mutants with reduced activity of dUTPase have significant uracil incorporated into DNA, but this is normally not mutagenic (*e.g.* (54)). However, under certain circumstances the additional uracil can be mutagenic, such as when a reduced activity dUTPase mutant also carries a mutation in the *ndk* gene (54). Wright and coworkers have argued that NDK is involved in removing uracil from DNA (54), although it could simply be that NDK-deficient cells are just beginning to saturate MMR, and the combination of NDK-deficiency and reduced dUTPase activity titrate out MMR, and the result is the higher error rate observed. It is possible that, in this scenario, under normal conditions in a wild-type strain a certain amount of uracil could get into the DNA and cause replication errors that are few enough to be corrected by mismatch repair, but are revealed in a MMR-deficient strain. Then, in a PNP-deficient strain, the reduced pools of rUDP would result in greatly reduced polymerase errors, and a reduced mutation frequency. Arguing against this, however, is our demonstration that for at least the main hotspot in *rpoB* the key intermediate is an A:C mispair, and not a G:T or G:U mispair (55).

Perhaps the most likely model involves the concept that the levels of the pools of all four canonical bases in DNA determines the rate of DNA replication (56). Several groups have shown that plasmid overexpression of RNR results in increased levels of dNTP's and increased mutagenesis (22, 23). Moreover, a deletion of the *cmk* gene in E. coli results in reductions in the dCTP pools (to 30% of the wild-type level) and the dTTP pools (to 70% of the wild-type level), and a subsequent two-fold reduction of the replication elongation rate (57). A number of studies have shown that in higher cells the nucleotide pools affect the speed of replication. In mammalian somatic cells a reduction in nucleotide availability results in a slowing of the rate of fork progression (58). In mice embryos, the rate of DNA synthesis is closely correlated with the intracellular deoxynucleoside pools during embryonic development (59). In synchronized populations of human HeLa cells, the addition of exogenous dNTPs accelerated the speed of

replication fork movement in early S-phase (60). Our results here suggest that in a wild-type strain, the dNDP pools emanating from rNDP's generated by PNP (Figure 4-6) generate replication errors that are repaired by the MMR repair system. Elimination of the additional rNDP pool created by PNP, by deleting the *pnp* gene, sharply reduces these errors, so that even in a MMR-deficient strain one does not observe mutations much over the background.

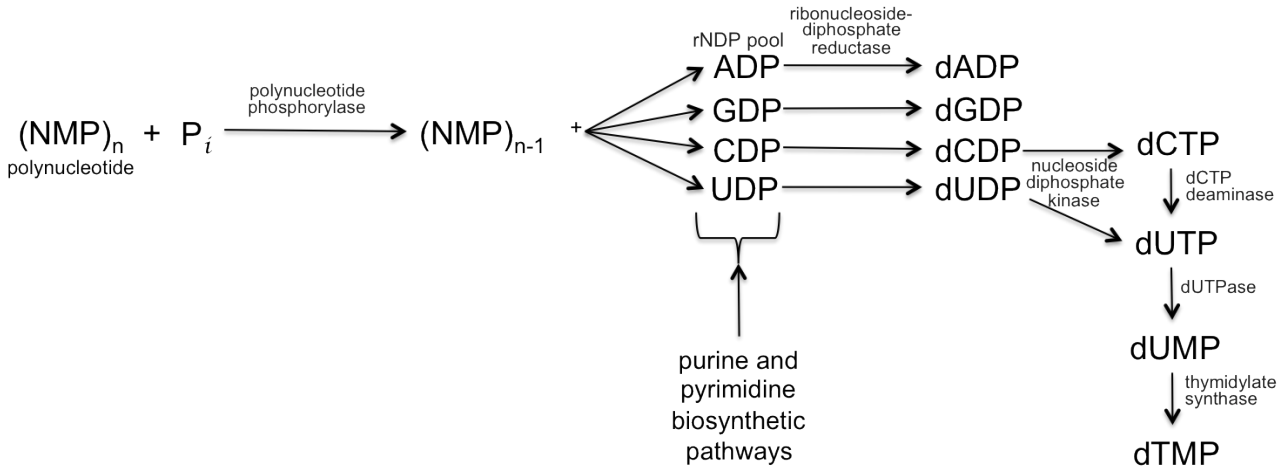

Figure 4-6. Pathways emanating from RNA biosynthesis and degradation. Adapted from (47, 61)

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CHAPTER 5: Exploring the Contribution of Polynucleotide Phosphorylase on the Spectrum of Mutations in *Escherichia coli*

Abstract

Polynucleotide phosphorylase (PNP) is an exoribonuclease whose activity has been shown to result in mutations normally repaired by the mismatch repair complex, and prevented by 8-oxo-dGTP diphosphatase. However, the mechanism of how PNP activity results in these mutations is not completely understood. Here we used knockout and overproducer strains to determine the pathway through which these mutations occur. Our findings suggest that the nucleoside pools produced by PNP affect replication speed and thus DNA polymerase fidelity. Our discovery that PNP-deficiency does not affect mutagenesis induced by and alkylating agent further suggests the responsibility of nucleoside pools in the mutations seen. Additionally, we discovered that PNP mutants are susceptible to frameshift mutations, and we analyzed the spectrum of these mutations using PCR and sequencing.

Introduction

Polynucleotide phosphorylase (PNP) is a phospholytic exonuclease that is involved in the degradation of RNA, creating a pool of rNDPs (1, 2) that combines with pools from *de novo* nucleoside biosynthesis (3, 4). This pool of nucleoside diphosphates can then be (re)incorporated into RNA, or they can be reduced by ribonucleoside diphosphate kinase to dNDPs that are phosphorylated by nucleoside diphosphate kinase and incorporated into DNA. However, *de novo* pyrimidine biosynthesis is carried out at a low level (1), and there is 10-fold more RNA in the in the cell than DNA (5), with ribonucleoside pools measured to greatly exceed deoxyribonucleoside pools (6). Thus the primary source of pyrimidines for DNA synthesis may

be the pool of nucleosides derived from RNA, implicating PNP-activity in generating a significant portion of nucleoside pools needed for DNA replication.

In the previous chapter, we demonstrated that the absence of PNP virtually eliminated mutations in 5BdU-treated cells and in cells deficient in mismatch repair (MutL) and 8-oxo-dGTP diphosphatase (MutT). We provided three explanations for how this occurs. The first explanation was that PNP activity produced ribonucleosides that were misincorporated into DNA, resulting in mutations (7-9). However, we disproved this mechanism by showing that a deficiency of either two enzymes responsible for degradation of RNA-DNA hybrids (RNase H1 or RNase H2) are not mutators in either an otherwise wild-type background or a MMR-deficient background. The two alternate explanations are: 1) mutations result from PNP-produced rUDP pools, which are then reduced and phosphorylated to dUTP (an intermediate required for the production of dTTP) and misincorporated into DNA, or 2) the dNTP pools emanating from rNDP's generated by PNP result in replication errors that escape editing from the ε subunit of polymerase III, then are repaired by the MMR repair system. In this chapter, we will explore these two latter explanations, as well as further explore the change in the spectrum of mutations from deficiency in PNP.

Materials and Methods

***E. coli* strains**. The Keio collection is as described in Baba *et al*. (10), made from the starting strain BW25113 (11). This strain (*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$) is the starting strain used in the experiments reported here, unless otherwise stated. Briefly, each of the 3,985 strains in the KEIO collection carries a complete deletion of a different gene, with a *kan* insert replacing each gene. The frameshift tester strain, CC107, has been described previously (12). It carries a frameshift mutation in *lacZ* on the F' plasmid, which reverts from Lac^{-}

to Lac$^+$ only by restoring the glutamic acid codon through a reversion by a specific frameshift mutation in a run of G's. Additionally, these strains carry a mini*Tn*10*cat* insert (13) conferring chloramphenicol resistance for selection purposes (C. Tamae and J. H. Miller, unpublished).

***E. coli* genetic methods.** Unless otherwise stated, all genetic methods are as described by Miller (13).

Validation controls. A collection of close to 4,000 strains will contain some errors, and some impure strains. The latter problem can be minimized by repurifying and retesting, as was done here. See Chapter 4 Material and Methods for details of validation controls done to verify the *pnp* strain used here.

Construction of *dut* overexpression vector – PCR primers (5' – CAGAGAAAATCAAAAAGCAGGC - 3'; 5' – GTTTGCGGCTATGTTATGACG - 3') were used to amplify the segment of DNA including the *dut* gene and it's endogenous promoter using Taq polymerase (Invitrogen). The PCR product was then ligated onto the TOPO XL vector using the protocol from the TOPO XL cloning kit (Invitrogen). After chemical transformation into One Shot® TOP10F' cells (Invitrogen), the cloned plasmids were then isolated using the PureLink^{TM} Quick Plasmid Miniprep Kit (Invitrogen) and digested with restriction enzyme EcoRI (Invitrogen). The QIAexpress pQE-60 vector (QIAGEN) was then digested using EcoRI (Invitrogen), disrupting the IPTG-inducible T5 promoter in the pQE-60 vector. This vector was then ligated to the digested fragment containing the *dut* gene with endogenous promoter (Figure 5-1A). DNA sequencing using primers for the pQE-60 plasmid was performed to verify the insert sequence and orientation.

Verification of *dut* overexpression – *E. coli* wild-type and *mutL* strains, containing pQE-60 vectors with or without the *dut* insert, were grown for 18 hours at 37°C with aeration. 50 x 10^8 cells (~15µl) were combined with equal volumes of 2X Novex® Tris-Glycine SDS sample buffer (Invitrogen), and heated at 95°C for 5 minutes. Samples were then loaded into the non-reducing Novex® NuPAGE® Tris-glycine SDS-PAGE gel (Invitrogen); Dut exists as a homotrimer held together by hydrogen bonds and not disulfide bonds, thus no reducing agent was needed. This gel was ran in the XCell *SureLock*™ Mini-Cell (Invitrogen), using Tris-glycine SDS sample buffer (Invitrogen) without sample reducing agent, at 125 volts for 90 minutes. The Bio-Rad Precision Plus™ protein ladder was used as a standard. The acrylamide gel was then removed from the cell and placed in staining buffer (0.25g coomassie brilliant blue R250 in 100ml of destaining buffer) for 40 minutes under gentle shaking conditions. The gel was then placed in destaining buffer (45% methanol, 45% ddH_2O, 10% glacial acetic acid) overnight under gentle shaking conditions. In both wild-type and mismatch repair-deficient strains used in later experiments, we verified that the construct with the insert produces the desired 16.1 kDa band, which is absent in the respective strains containing the empty vector (Figure 5-1B).

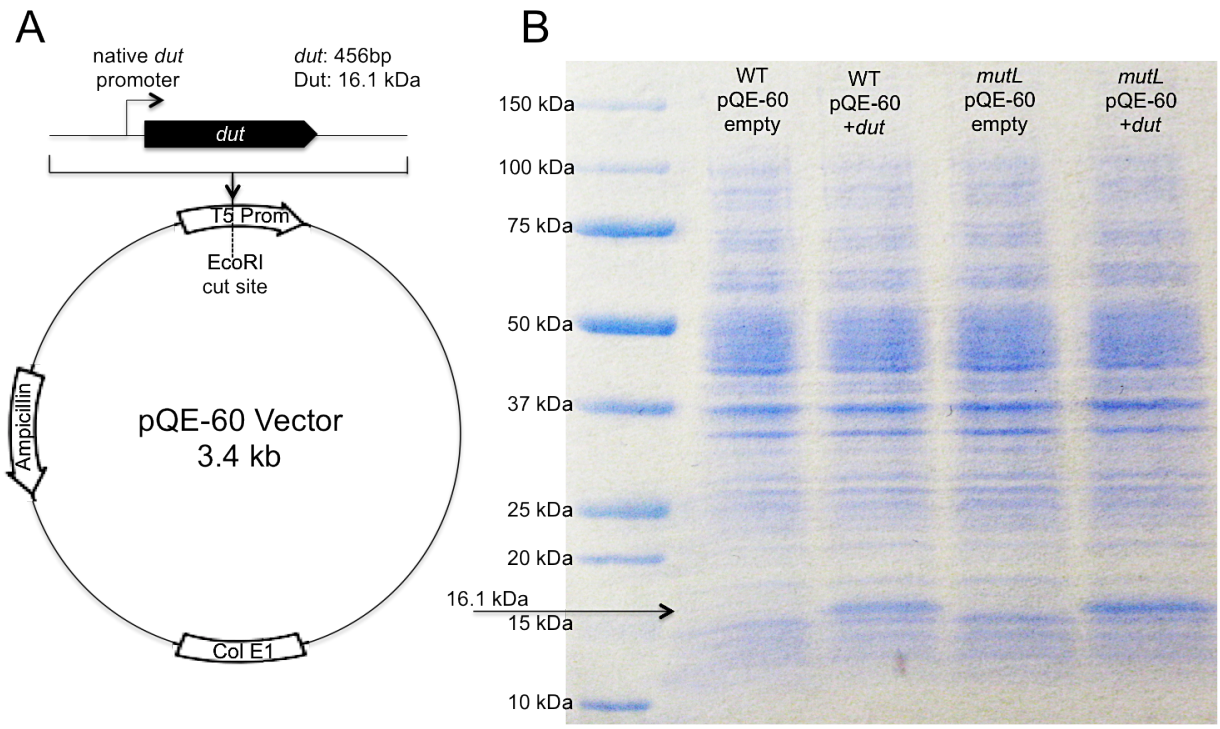

Figure 5-1. Construction and verification of the *dut* overexpression vector.

Determination of mutation frequencies - Mutagenesis with ethyl methanesulfonate (EMS) was carried out as described in Miller (13) and Cupples and Miller (14). The mutation frequencies of Rif^r and Lac^+ revertants were determined as described previously (15). Briefly, mutation frequency (f) was determined as the median frequency from a set of cultures (the number of cultures varied from 8 to 20), and the mutation rate (μ) was determined by the formula of Drake (16). 95% confidence limits were determined according to the method of Dixon and Massey (17).

Numbering system for rpoB and gyrA mutations – The nucleotide sequences for the *E. coli rpoB* and *gyrA* genes were obtained from the NCBI online database (accession number NC_000913.2). The numbering system begins at the start codon, ATG, with A labeled as #1.

PCR and sequencing of *rpoB* and *gyrA* mutants – Wild-type and *pnp* strains were plated on Rif and Nal to obtain resistant mutants. Single colonies of Rif^r and Nal^r mutants were repurified on LB agar plates and then picked into PCR reaction tubes to amplify the *rpoB* and *gyrA* genes, respectively. The primers for *rpoB* were 5′-CGTCGTATCCGTTCCGTTGG-3′ and 5′-TTCACCCGGATAACATCTCGTC-3′ as described in (18). The first 350 bp of *gyrA*, where the majority of mutations have been found to occur, was amplified using the primers 5'-AAACGAGTATATCAGGCATTGG-3' and 5'GCAGAGTCGCCGTCGATAG-3' as described in (19). Amplification was performed using Lambda Biotech TSG Polymerase. PCR products were subsequently treated with shrimp alkaline phosphatase (SAP) and exonuclease I (ExoI) from Lambda Biotech. Amplicons were then sequenced and submitted to the UCLA Genotyping and Sequencing Core Facility for sequence readings. The sequencing primer for the *rpoB* gene was 5′-CGTGTAGAGCGTGCGGTGAAA-3′, and that for the *gyrA* gene was 5'-AAACGAGTATATCAGGCATTGG-3'. The resulting sequences were then aligned to a reference sequence of *rpoB* and *gyrA* derived from the NCBI database and using the program SeqMan in order to identify the location and nature of the mutation.

If no mutation occurred in the *rpoB* region initially sequenced, another fragment of the *rpoB* gene, where a significant minority of mutations also occur, was amplified using the primers 5′-AATGCAAATCCGTGGCGT-3′ and 5′-CCAACCGCAGACAAGTCATA-3′. After treatment with SAP and ExoI, samples were sequenced with the primer 5′-GAAGGCACCGTAAAAAGACAT-3′ as described in (18). Samples were processed and analyzed as described above.

If no mutation occurred in the *gyrA* region initially sequenced, the entire *gyrA* gene was amplified to determine the location of the mutation. Due to the large size of *gyrA*, three sets of primers were designed to amplify the gene in three overlapping fragments. The gene was amplified using the primer sets 5'-GGGATACAGTAGAGGGATAGCG-3' and 5'-TTGCAGGTTTCTTTCGGTATC-3'; 5'-CAGGTAAACAAAGCGCGC-3' and 5'-TCGTCAACCTGCTGCCG-3'; and 5'-GCCGCACGTATTAAAGAAGAA-3' and 5'-

CGACGAGCCAGAAGAAGAATA-3'. Products were treated with SAP and ExoI. Samples were sequenced using the corresponding forward primer for each set and processed by the UCLA Genotyping and Sequencing Core. The three sequences were then simultaneously aligned to the reference sequence of gyrA using SeqMan in order to determine the location and nature of the mutation.

PCR and sequencing of *thyA* mutants – Wild-type and *pnp* strains were grown overnight in LB supplemented with thymidine at 37°C. They were then plated on glucose minimal medium with thymidine and trimethoprim (TMP). TMP^r colonies then underwent PCR and sequencing analysis as described in the section above, using PCR primers 5' - GGTGTGATCATGATGGTCTGG - 3' and 5' - CACACTGGCGTCGGCTC - 3', using these primers for sequencing as well.

Chemicals – Rifampicin, nalidixic acid, tetracycline, chloramphenicol, and kanamycin were purchased from Sigma (St. Louis, MO).

Results

Activity of dUTPase does not contribute to mutations repaired by mismatch repair – In an earlier chapter we demonstrated that deletion of *pnp* reverses the mutations seen in a strain deficient in mismatch repair (MMR). Thus we sought to determine if the mutations resulting from PNPase activity were from uracil misincorporation in DNA. We overexpressed dUTPase with its native promoter on a low-copy plasmid (see Materials and Methods), and transformed this plasmid into a *mutL* strain (MMR^-), using the strain with the empty vector as a control. We did not observe a significant difference in mutant frequency between these two strains.

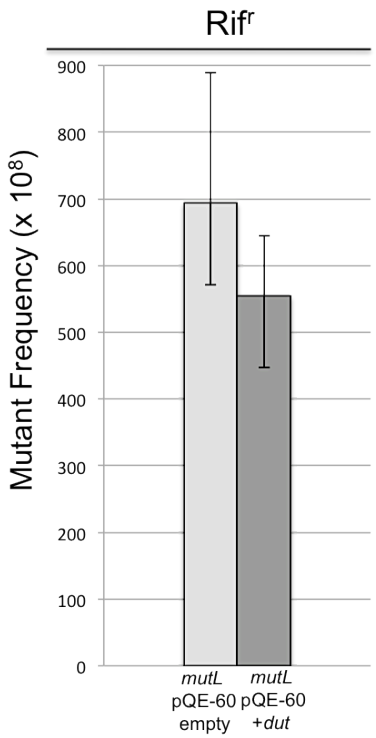

Figure 5-2. Mutagenesis in *mutL* strains containing an empty pQE-60 vector and vector with *dut* insert. Mutant frequencies for spontaneous mutations in *rpoB*, generating Rif^r mutants, are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details.

Polymerase III editing function repairs replication errors resulting from PNP activity – To demonstrate whether PNP activity results in replication errors repaired by Pol III's editing subunit, we compared the mutant frequencies between a strain deficient in this editing subunit (*dnaQ*) and a double knockout strain of *dnaQ* and *pnp*. Using assays analyzing for Rif^r and Nal^r mutants, we found a 10-fold and 7.8-fold decrease, respectively in the double knockout with respect to the single knockout mutant (Figure 5-3). This implicates PNP in the occurrence of mutations normally repaired by Pol III's editing subunit.

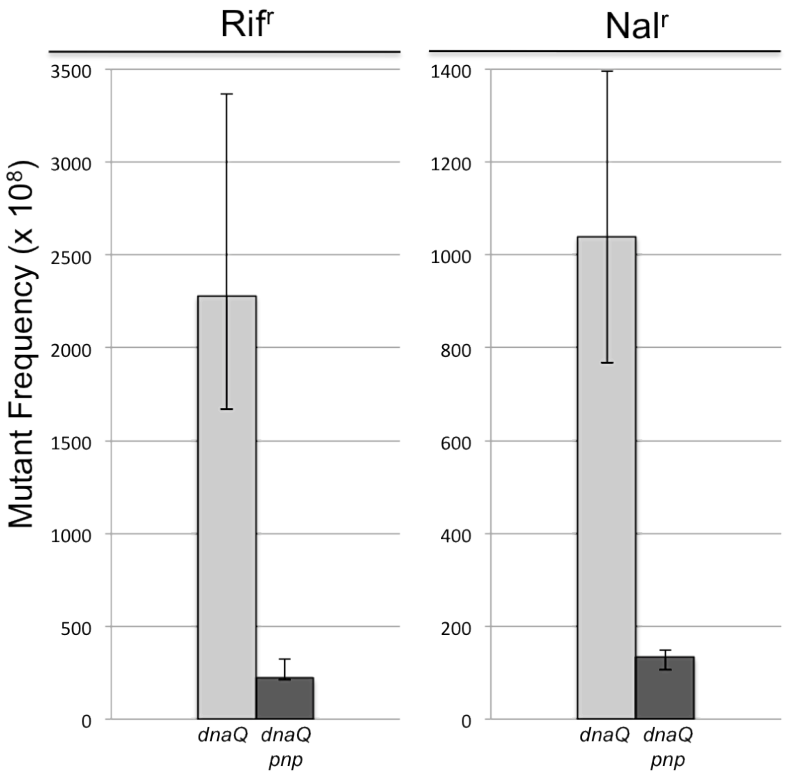

Figure 5-3. Mutagenesis in *dnaQ* single knockout and *dnaQ pnp* double knockout strains. Mutant frequencies for spontaneous mutations in *rpoB*, generating Rif^r mutants, and *gyrA*, generating Nal^r strains, are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details.

Mutagenesis in *pnp* strains in response to alkylation and frameshift inducers – In the previous two chapters, we analyzed mutations in a PNP-deficient strain induced by base analog mutagens (5-azacytidine and 5-bromodeoxyuridine). To further understand the types of mutations seen in a *pnp* strain, we tested the mutant frequencies in a wild-type and *pnp* strain induced by the alkylating agent ethyl methanesulfonate (EMS; (20)). We did not find any significant change in EMS-mediated mutagenesis in the *pnp* versus wild-type strain (Figure 5-4), indicating that mutagenizing DNA via alkylation is unaffected by PNP-deficiency.

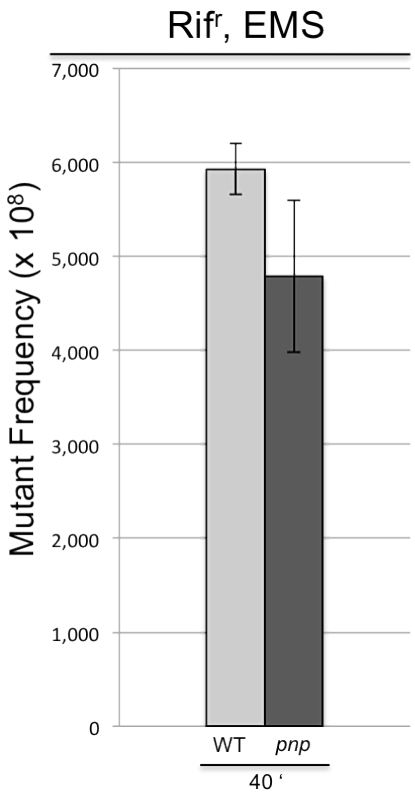

Figure 5-4. Mutagenesis in WT and PNP-deficient strains. Mutant frequencies for EMS (40-minute treatment)-induced mutations in *rpoB*, generating Rif^r mutants are shown. Error bars reflect 95% confidence limits. See Materials and Methods for further details.

Spectrum of mutations in a PNP-deficient strain – The spectrum of mutations in individual strains and conditions reveal characteristic patterns of hotspots and coldspots (18, 21-23). Here we analyzed the spectrum of mutations in the *rpoB* and *gyrA* genes that lead to a Rif^r (18) and Nal^r ((19), see Chapter 4) phenotype, respectively, to see if the absence of PNP has an effect on the mutational spectrum seen in the wild-type strain. We did not discover a notable change in the representation of base-substitutions between the two strains, but we discovered a set of deletions in both the *rpoB* and *gyrA* genes in the *pnp* strain. Such deletions are very rare and not found by previous studies in either the *rpoB* or *gyrA* genes ((18, 24, 25), Chapter 4).

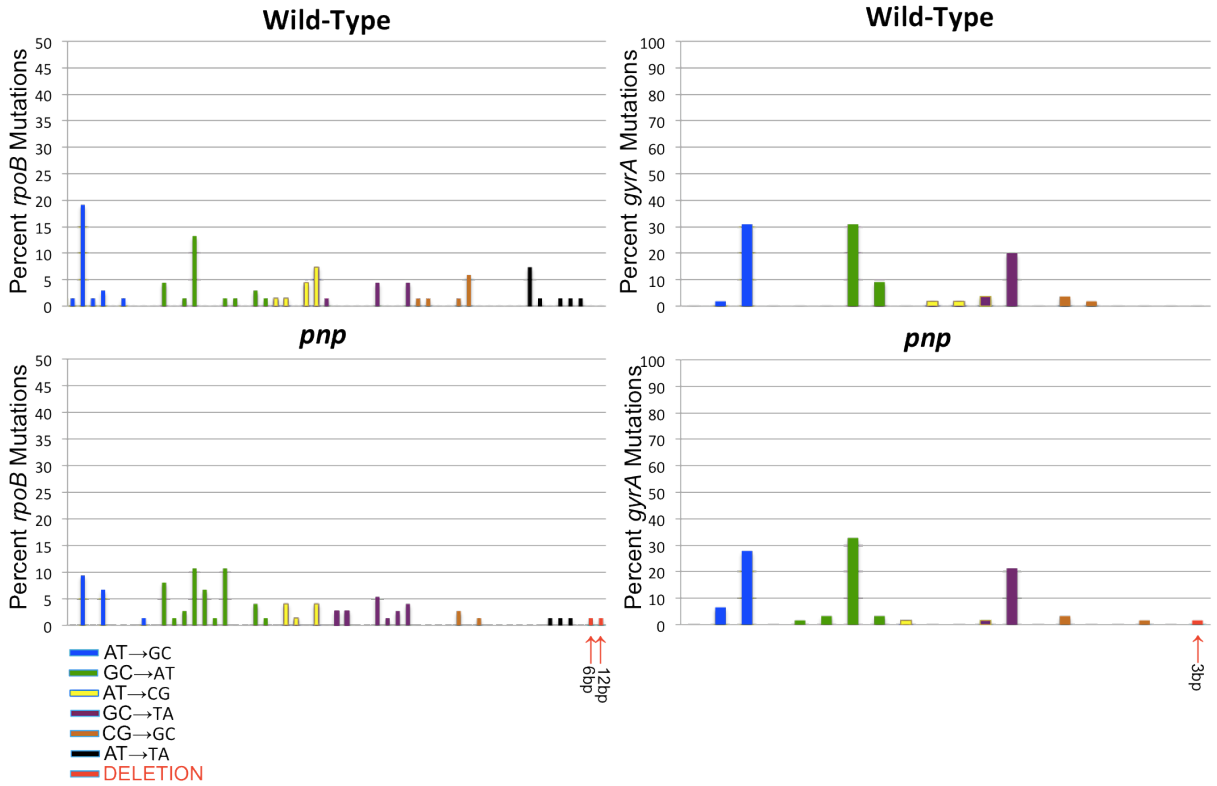

Figure 5-5. Transition mutations in *rpoB* and *gyrA* leading to the Rif^r or Nal^r phenotype occurring spontaneously. Types of mutations are color-coded in each diagram, according to the legend, and placed in order of mutation type then gene location. The height of each bar represents the percentage of all the *rpoB* or *gyrA* mutations detected in that sample. For further details consult the text.

Deficiency in PNP results in increased insertions and deletions – Since *rpoB* and *gyrA* are essential genes (26, 27), only a limited number of insertions or deletions could occur without disrupting the gene function and losing cell viability. Thus, we needed a system to detect mutations that would allow a more extensive analysis of insertions or deletions, such as the *thyA* gene. When supplemented with thymidine, cells become trimethoprim-resistant when the *thyA* gene is disrupted (28). We analyzed the spectrum of spontaneous mutations occurring in the *thyA* gene (as done with *rpoB* and *gyrA,* and described in Materials and Methods) in a wild-type and *pnp* strain. There were two major mutational hotspots, a T:A→A:T transversion at position 131 and a 6-bp insertion/deletion at position 64, and a minor mutational hotspot of a 6-bp deletion at position 62. The full spectrum of mutations is diagrammed in Figure A-5-1. We

discovered that while the occurrence of the transversion hotspot mutation greatly outweighs the occurrence of the hotspot insertion/deletion in the wild-type strain, the representation of the insertion/deletion in the *pnp* strain is greatly increased (Figure 5-6). The wild-type strain reveals only 10% of mutations in these hotspots and 12% in all *thyA* sites are insertions or deletion. The *pnp* strain demonstrates 49% of hotspot mutations and 44% of total *thyA* mutations are insertions or deletions. Additionally, there were more total types of mutations in *thyA* seen in the *pnp* strain (57/140 sequenced, at 54 sites), than in the wild-type strain (31/145 sequenced, at 29 sites).

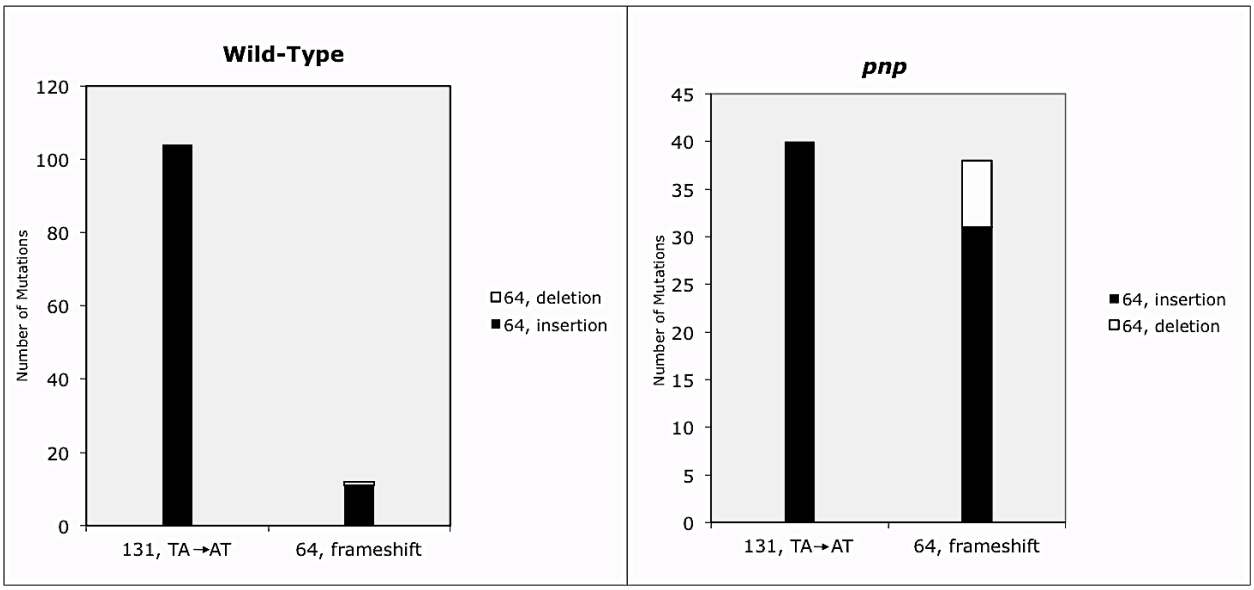

Figure 5-6. Number of hotspot base-substitution mutations and insertion/deletions in the wild-type and PNP-deficient strains. The number of TMP^r colonies showing mutations in either of two mutational hotspots in the *thyA* gene (position 131, transversion; position 64, insertion or deletion) are represented here.

Discussion

In this chapter, we sought to elucidate the mechanism through which PNP activity results in mutations, as well as the spectrum of induced and spontaneous mutations seen in a PNP-deficient strain. First, we tested if rUDP pools produced by PNP are responsible for the mutagenesis resulting from PNP activity. As described earlier, rUDP is converted by ribonucleoside diphosphate reductase to dUDP, and then converted to dUTP by nucleoside diphosphate kinase. This dUTP is an essential intermediate in the production of dTTP for DNA incorporation. However, dUTP can be misincorporated into DNA, resulting in mutagenic A:U lesions (29). dUTPase converts dUTP to dUMP, thereby depleting dUTP pools and preventing its utilization by DNA polymerases during replication and repair (30). However, while mutants with reduced activity of dUTPase have significant uracil incorporated into DNA, studies have not found an increase in mutations in this strain (31, 32). However, under certain circumstances the additional uracil can be mutagenic, such as when a reduced activity dUTPase mutant also carries a mutation in the *ndk* gene (31). In this study, we discovered that the increased removal of dUTP pools in a PNP-proficient strain does not significantly suppress mutations seen in a MMR^- strain. In the previous chapter we observed that PNP-deficiency virtually eliminates the mutations seen in a MMR^- strain, thus the mechanism through which PNP reverses this mutation is not through the production of uridine nucleosides that are misincorporated into DNA.

Our data supports our alternate theory however, that the pool size of all four DNA nucleosides affects the rate of replication and thus replication errors are missed by the Pol III editing subunit. Several studies have demonstrated that overexpression of ribonucleoside diphosphate reductase, resulting in the increase of all four canonical dNTPs, also results in increased mutagenesis (33, 34). Additionally, several studies have shown in bacteria and higher cells that reducing nucleotide pools also decreases the speed of replication (35-37), and increasing the pools accelerates replication fork movement (38). There is 10-fold more RNA in

the in the cell than DNA (5), thus PNP's activity in RNA turnover contributes significantly to the nucleoside pools in the cell. It is therefore likely that the pools generated by PNP increase dNTP pools, speeding up replication. Further demonstrating this link, studies have shown viruses that replicate faster within host cells also have higher mutation rates, thus there is a tradeoff in speed versus accuracy of replication (39). This tradeoff has also been hypothesized in mutli-cellular eukaryotes (40). To test this link, we measured if PNP-deficiency reverses mutations seen in a strain lacking in the Pol III editing subunit (*dnaQ*), and we found that a lack of PNP does indeed decrease mutations in *dnaQ* by 8-to-10-fold (Figure 5-3). Collectively, previous studies combined with our results, in this and the previous chapter, suggest that PNP activity increases nucleoside pools, thus increasing replication speed and in turn replication errors normally repaired by DnaQ and MMR. Further studies include measuring the pools of rNDPs and dNTPs in a wild-type and *pnp* strain to validate this model.

Our data showing that PNP activity does not affect EMS-mediated mutagenesis supports the theory that rNDP pools produced by PNP are influencing mutagenesis. EMS is an alkylating agent reacting directly with nucleotides in DNA, producing ethylated nucleotides (41), and resulting in G:C→A:T mutations (14, 18). This direct interaction with bases already in DNA instead of nucleoside pools makes it logical that we found no significant change in EMS-mediated mutagenesis in the *pnp* strain versus wild-type.

Our data regarding the spectrum of mutations also reveals that *pnp* strains are somehow more prone to insertions or deletions. In only the *pnp* strain we observed a set of deletions in the mutations spectra of *rpoB* and *gyrA* (a 6-bp and 12-bp deletion in *rpoB*; a 3-bp deletion in *gyrA*) (Figure 5-5). Deletions in these genes when analyzing mutational spectra have not been observed in other strains in previous studies, even with hundreds of mutants sequenced ((18, 24, 25), and Chapter 4). While this number of deletions is not high, *rpoB* and *gyrA* are essential genes (26, 27), and thus can only undergo a limited number of mutations, particularly insertions/deletions, to maintain gene function. Thus we needed a mutation detection system,

like TMP^r resulting from *thyA* disruption, in which gene disruption results in a mutant phenotype, allowing a much broader range of mutations to be detected. We discovered that while both wild-type and *pnp* strains produce insertion and deletion mutations in this system, *pnp* does so much more frequently (Figure 5-6). The wild-type strain reveals only 10% of mutations in the two hotspots and 12% in all *thyA* sites are insertions or deletion. The *pnp* strain demonstrates that 49% of hotspot mutations and 44% of total *thyA* mutations are insertions or deletions. The increase in these insertions and deletions in the *pnp* strain is curious; it is possible that the loss of PNP activity is lowering the occurrence of predominant replication errors (as demonstrated in Chapter 4), thus revealing errors normally less prevalent. Evidence supporting this is the finding that there were more total types of mutations in *thyA* seen in the *pnp* strain (57 mutations/140 revertants sequenced, at 54 sites), than in the wild-type strain (31 mutations/145 revertants sequenced, at 29 sites) (Figure A-5-1). Combined with the discovery that PNP virtually eliminates mutations in MMR-deficient and MutT-deficient cells, these data suggest that the *pnp* strain reveals mutations normally masked by the predominant errors in normal conditions.

CHAPTER 5 Bibliography

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APPENDIX

Bleomycin

Cat.	Gene	MIC
	BW25113	1.25
1	*dinB*	0.5
1	*recA*	0.156
1	*recB*	>0.156
1	*recC*	0.156
1	*recG*	0.313
1	*recN*	0.313
1	*ruvA*	0.5
1	*xerD*	0.5
1,6	*ybcN*	0.313
1A	*fis*	0.5
1A	*ftsP*	0.313
1A	*rnhA*	0.5
2	*acrA*	0.156
2	*acrB*	0.156
2	*tolC*	0.156
2	*bamB*	0.156
2	*ddlB*	0.5
2	*dedD*	0.156
2	*envC*	0.156
2	*fepC*	0.5
2	*fhuA*	>0.5
2	*lpxL*	0.156
2	*lpxM*	0.313
2	*mrcB*	0.5
2	*ppiD*	0.5
2	*pstS*	0.5
2	*rfaC*	0.313
2	*rfaD*	0.313
2	*rfaE*	0.156
2	*smpA*	0.313
2	*tatB*	>0.5
2	*tatC*	0.156
2	*tolQ*	0.5
2	*tolR*	0.313
2	*ybgF*	0.5
2	*tonB*	0.5
2	*ybjL*	0.5
2	*yciB*	0.5
2	*ydcS*	0.5
2A	*dnaK*	0.313
2A	*hlpA*	0.156
2A	*surA*	0.156
3	*elaD*	0.5
3	*rplK*	>0.5
3	*rpmF*	0.5
3	*yfgC*	0.313
3A	*pcnB*	0.5
3A	*rppH*	>0.313
4	*fliI*	0.5
4	*folB*	0.5
4	*yqfA*	0.5
4	*qmhB*	0.313
4	*qpmM*	0.156
4	*metL*	0.5
4	*nagA*	0.5
4	*nudB*	0.313
4	*pgmB*	0.5
4	*ubiG*	>0.5
4	*ybgC*	0.5
4	*ycjU*	0.5
4	*yqcO*	0.5
4	*ytjC*	0.5
4	*zwf*	0.5
5	*dksA*	0.313
5	*fur*	0.313
5	*qcvA*	0.5
5	*hfq*	0.156
5	*rseA*	0.5
5	*xapR*	0.5
5	*yciT*	0.5
5,6	*ymfT*	>0.156
5	*zapB*	0.5
6	*ylcG*	0.313
7	*JW5015*	0.5
7	*JW5115*	0.5
7	*JW5360*	>0.5
7	*ybeD*	0.5
7	*ybfJ*	0.5
7	*ybhT*	0.5
7	*ycbW*	0.313
7	*yceD*	0.5
7	*ychJ*	0.5
7	*yciM*	0.156
7	*yfiH*	0.5
7	*yjdD*	0.5

Cisplatin

Cat.	Gene	MIC
	BW25113	250
1	*dam*	200
1	*recA*	50
1	*recB*	100
1	*recC*	100
1	*recG*	100
1	*recN*	200
1	*recF*	150
1	*recO*	200
1	*recR*	200
1	*ruvA*	150
1	*ruvC*	>200
1	*uvrA*	100
1	*uvrB*	150
1	*uvrC*	150
1	*uvrD*	100
1	*xerC*	200
1	*xseA*	>200
1	*xseB*	200
1A	*cmk*	200
1A	*fis*	200
1A	*ftsP*	200
1A	*gshA*	200
1A	*gshB*	200
1A	*rnhA*	>200
2	*acrA*	200
2	*tolC*	150
2	*ddlB*	200
2	*dedD*	150
2	*envC*	150
2	*envZ*	200
2	*fepC*	150
2	*lpxL*	150
2	*lpxM*	150
2	*ompF*	200
2	*plsX*	200
2	*pstC*	200
2	*pstS*	200
2	*rfaC*	150
2	*rfaD*	150
2	*rfaE*	150
2	*rfaG*	150
2	*rfaP*	150
2	*sapC*	200
2	*tatB*	200
2	*tatC*	150
2	*tolQ*	200
2	*yicL*	200
2	*yneE*	>200
2A	*dnaK*	200
3	*rpmF*	200
3	*yfgC*	150
3	*yheN*	200
3A	*rppH*	100
4	*aceF*	150
4	*ackA*	200
4	*pta*	200
4	*atpG*	200
4	*dapF*	150
4	*fabF*	200
4	*folB*	200
4	*folP*	200
4	*ygfA*	200
4	*qmhB*	200
4	*qpmM*	150
4	*nagA*	200
4	*nudB*	150
4	*pgm*	200
4	*rpe*	200
4	*rpiA*	200
4	*ybgC*	200
4	*yciU*	200
4	*ydbD*	200
4	*yqfZ*	200
4	*yigB*	200
4	*ytjC*	200
5	*deaD*	200
5	*qcvA*	150
5	*hfq*	150
5	*oxyR*	200
5	*rfaH*	150
5	*rseA*	200
5	*xapR*	150
5	*ymfT*	100
6	*ylcG*	200
7	*ybaB*	200
7	*ybeD*	200
7	*ybhT*	200
7	*ychJ*	200
7	*yciM*	150
7	*yjiY*	200

ICR-191

Cat.	Gene	MIC
	BW25113	>30
1	*dam*	>20
1	*recA*	8
1	*recB*	>20
1	*recC*	20
1	*recG*	20
1	*ruvA*	12
1	*uvrA*	20
1	*uvrC*	>20
1	*uvrD*	>20
1	*xseB*	>20
2	*acrA*	10
2	*acrB*	10
2	*tolC*	8
2	*dedD*	10
2	*envC*	>20
2	*lpxL*	12
2	*rfaC*	15
2	*rfaD*	12
2	*rfaE*	12
2	*rfaG*	15
2	*tatC*	>>20
2A	*dnaK*	>20
4	*pta*	>20
4	*qmhB*	12
4	*qpmM*	>20
4	*iscS*	>20
4	*rpe*	20
4	*ydbD*	15
5	*hfq*	20
5	*oxyR*	20
5	*rfaH*	>10
5,6	*ymfT*	>20
7	*yciM*	20

5-Azacytidine

Cat.	Gene	MIC
	BW25113	>200
1	*dam*	>100
1	*recA*	80
1	*recB*	30
1	*recC*	30
1	*recG*	80
1	*ruvA*	80
1	*ruvC*	>150
1	*uvrD*	100
1	*xthA*	>100
1A	*fis*	60
1A	*rnhA*	100
2	*crr*	>150
2	*dcuC*	>100
2	*dedD*	100
2	*envC*	>100
2	*fepG*	>150
2	*lpxL*	>100
2	*tatB*	100
2	*tatC*	100
2A	*dnaK*	100
3	*rplA*	80
3	*rpmJ*	80
3	*rpsF*	>100
3A	*rhlB*	>150
4	*aceE*	>100
4	*aceF*	>100
4	*ackA*	>>100
4	*pta*	>150
4	*dapF*	>100
4	*qpmM*	100
4	*iscS*	>100
4	*lpdA*	100
4	*upp*	100
5	*nusB*	150
5	*oxyR*	>100
5	*rseA*	>150
6	*racC*	>100
7	*JW3133*	150
7	*JW5115*	100
7	*ybeY*	80
7	*yddK*	>100

Zebularine

Cat.	Gene	MIC
	BW25113	150
1	*dam*	>100
1	*recA*	>60
1	*recB*	<100
1	*recC*	60
1	*recG*	100
1	*recN*	100
1	*ruvA*	>80
1	*uvrC*	>100
1	*uvrD*	100
1	*xerC*	100
1	*xerD*	>80
1	*xseA*	>100
1	*xthA*	>100
1A	*rnhA*	>100
1A	*tdk*	60
2	*dcuC*	<100
2	*dedD*	>100
2	*envC*	>100
2	*lpxL*	60
2	*lpxM*	60
2	*plsX*	60
2	*tatC*	100
2A	*degP*	100
2A	*dnaK*	>80
2A	*surA*	100
3	*rplA*	80
3	*rpmF*	100
3	*rpmJ*	60
3	*rpsF*	>100
3	*sirA*	>100
3	*yfgC*	>100
3	*yheM*	>100
3	*yheN*	>80
3A	*pcnB*	80
3A	*pnp*	>100
4	*fabF*	100
4	*fliI*	>100
4	*folP*	>100
4	*ygfA*	60
4	*iscS*	100
4	*metL*	>100
4	*nudB*	100
4	*upp*	>100
4	*ybgC*	>80
4	*yebR*	100
4	*zwf*	<100
5	*oxyR*	80
5	*xapR*	>100
6	*ydfP*	100
7	*JW5474*	80
7	*ybeY*	60
7	*ycbK*	>100
7	*ychJ*	>80
7	*yciM*	>100
7	*yhcB*	60
7	*yjiJ*	>100

5-Bromouracil

Cat.	Gene	MIC
	BW25113	>1000
1	*dam*	50

MIC = µg/ml

Figure A-2-1. Minimum inhibitory concentrations (MICs) of all hypersensitive strains, as determined by eye. After screening the entire KEIO collection, the hypersensitive strains were repurified and condensed onto five 96-well micro-titer plates. These strains were then retested against varying concentrations of each agent, from which the MICs for each mutant were determined. These values are listed here according to the agent, then subdivided into gene categories, as in Figure 2.

Gene	Bleo .313	CPT 150	ICR 20	5AZ 80	Zeb 100	5BU	Categ	Gene	Bleo .313	CPT 150	ICR 20	5AZ 80	Zeb 100	5BU	Categ
dam	-	13285.882	15455.163	24512.729	14433.438	49473.21	1	rpmF	-	8406.209	-	-	15676.68	-	3
dinB	10951.068	-	-	-	-	-	1	rpmJ	-	-	-	8652.981	14436.145	-	3
recA	18691.601	18599.666	18715.125	15368.078	18863.694	-	1	rpsF	-	-	-	14609.959	7634.87	-	3
recB	8417.695	16461.263	8756.341	11376.575	18860.577	-	1	sirA	-	-	-	-	18606.264	-	3
recC	12176.251	20492.723	15346.97	19908.663	18863.87	-	1	yfgC	13736.145	16765.338	-	-	14420.145	-	3
recG	11646.837	18535.301	20654.372	27229.029	18863.577	-	1	yheM	-	-	-	-	17333.837	-	3
recN	15869.556	8770.26	-	-	18865.577	-	1	yheN	-	11054.013	-	-	18865.87	-	3
recO	-	13842.937	-	-	-	-	1	pnp	-	-	-	-	12566.226	-	3A
recR	-	27471.339	-	-	-	-	1	rppH	14327.813	21714.815	-	-	-	-	3A
ruvA	7279.422	19156.268	18627.915	16794.269	18856.987	-	1	aceF	-	12510.469	-	10686.141	-	-	4
ruvC	-	7076.36	-	7003.053	-	-	1	ackA	-	19541.502	-	8066.814	-	-	4
uvrA	-	23935.108	12739.873	-	-	-	1	pta	-	26509.105	14467.566	8653.97	-	-	4
uvrB	-	27481.653	-	-	-	-	1	atpG	-	15527.121	-	-	-	-	4
uvrC	-	30064.452	10818.54	-	10861.718	-	1	dapF	-	21180.522	-	9843.298	-	-	4
uvrD	-	30559.229	11507.242	23670.984	13762.673	-	1	fabF	-	18142.595	-	-	15677.973	-	4
xerC	-	7182.721	-	-	29058.29	-	1	fliI	7955.035	-	-	-	12515.348	-	4
xerD	13093.264	-	-	-	18849.573	-	1	folB	-	7695.276	-	-	-	-	4
xseA	-	15977.097	-	-	-	-	1	folP	-	22653.603	-	-	18841.28	-	4
xseB	-	16033.807	8234.15	-	-	-	1	nudB	13895.313	29061.28	-	-	18867.577	-	4
xthA	-	-	-	8672.34	12704.54	-	1	yqfA	7741.64	17862.403	-	-	15671.973	-	4
ybcN	14316.473	-	-	-	-	-	1,6	gmhB	9838.89	25278.552	17123.467	-	-	-	4
cmk	-	26225.005	-	-	-	-	1A	gpmM	17231.39	18604.373	18461.216	11956.318	-	-	4
fis	15347.106	16482.85	-	20806.204	-	-	1A	iscS	-	-	9653.476	14048.763	18864.284	-	4
ftsP	10881.139	17381.854	-	-	-	-	1A	lpdA	-	-	-	7783.56	-	-	4
gshA	-	16145.414	-	-	-	-	1A	metL	-	-	-	-	10542.62	-	4
gshB	-	16449.677	-	-	-	-	1A	nagA	8053.745	11445.541	-	-	-	-	4
rnhA	8047.763	8448.758	-	9780.307	13194.084	-	1A	pgm	-	28684.23	-	-	-	-	4
tdk	-	-	-	-	29056.876	-	1A	pgmB	8313.179	-	-	-	-	-	4
acrA	21672.743	7606.569	15692.542	-	-	-	2	rpe	-	20458.188	21250.753	-	-	-	4
acrB	20895.618	-	14985.724	-	-	-	2	rpiA	-	31126.593	-	-	-	-	4
tolC	21130.471	26351.401	22922.636	-	-	-	2	upp	-	-	-	14562.872	13460.348	-	4
bamB	9659.162	-	-	-	-	-	2	ybgC	9715.172	8595.373	-	-	15674.68	-	4
dcuC	-	-	-	25308.159	29053.583	-	2	yciU	12508.618	17943.853	-	-	-	-	4
ddlB	11895.929	12186.038	-	-	-	-	2	ydbD	-	9223.9	8109.414	-	-	-	4
dedD	23873.627	23004.351	17391.574	25310.352	9211.363	-	2	yebR	-	-	-	-	15667.973	-	4
envC	19569.112	24235.51	8962.345	14020.742	22899.316	-	2	ygcO	22420.522	-	-	-	-	-	4
envZ	-	14179.544	-	-	-	-	2	yqfZ	-	12152.96	-	-	-	-	4
fepC	9247.165	16447.556	-	-	-	-	2	yiqB	-	11432.498	-	-	-	-	4
lpxL	19109.819	19625.469	18647.934	7097.247	19069.704	-	2	ytjC	8763.247	21311.886	-	-	-	-	4
lpxM	8685.151	16453.556	-	-	18754.72	-	2	zwf	-	-	-	-	18862.987	-	4
mrcB	8816.402	-	-	-	-	-	2	deaD	-	21805.644	-	-	-	-	5
ompF	-	14433.393	-	-	-	-	2	dksA	19065.715	-	-	-	-	-	5
plsX	-	33112.987	-	-	28791.441	-	2	gcvA	9762.672	20857.886	-	-	-	-	5
ppiD	9706.544	-	-	-	-	-	2	hfq	20793.555	21303.886	13230.465	-	-	-	5
pstC	-	19514.305	-	-	-	-	2	nusB	-	-	-	15154.376	-	-	5
pstS	-	16448.263	-	-	-	-	2	oxyR	-	13316.732	12562.97	16704.616	18839.916	-	5
rfaC	32537.299	32714.158	32010.173	-	-	-	2	rfaH	-	21317.886	26804.847	-	-	-	5
rfaD	16065.334	27713.865	20771.849	-	-	-	2	rseA	7877.69	18898.309	-	9115.485	-	-	5
rfaE	24358.835	29128.037	29505.336	-	-	-	2	xapR	9287.983	21238.351	-	-	-	-	5
rfaG	-	17603.348	19242.543	-	-	-	2	yciT	22926	-	-	-	-	-	5
rfaP	-	21309.593	-	-	-	-	2	ymfT	8219.964	18300.259	8617.281	-	-	-	5,6
sapC	-	24577.967	-	-	-	-	2	racC	-	-	-	8384.243	-	-	6
smpA	19528.758	-	-	-	-	-	2	ydfP	-	-	-	-	15603.609	-	6
tatB	8710.742	16707.824	-	23808.888	-	-	2	yjcG	10567.138	15707.713	-	-	-	-	6
tatC	8778.13	16467.677	8311.238	26244.352	18638.167	-	2	JW5015	8553.157	-	-	-	-	-	7
tolQ	-	21319.3	-	-	-	-	2	JW5115	-	-	-	7523.971	-		7
tolR	17918.82	-	-	-	-	-	2	JW5474	-	-	-	-	15680.387	-	7
tonB	12348.477	-	-	-	-	-	2	ybaB	-	16756.104	-	-	-	-	7
ybjL	9063.285	-	-	-	-	-	2	ybeD	-	17260.746	-	-	-	-	7
yciB	7858.935	-	-	-	-	-	2	ybeY	-	-	-	12341.597	15677.266	-	7
yicL	-	20948.522	-	-	-	-	2	ybhT	10464.129	18185.595	-	-	-	-	7
yneE	-	25833.967	-	-	-	-	2	ycbW	18786.285	-	-	-	-	-	7
degP	-	-	-	-	18861.284	-	2A	yceD	11216.292	-	-	-	-	-	7
dnaK	15141.313	12313.331	14983.653	13711.57	18588.071	-	2A	ychJ	11933.594	13265.654	-	-	15674.973	-	7
hlpA	16353.175	-	-	-	-	-	2A	yciM	18247.421	21323.3	30801.992	-	14236.468	-	7
surA	13813.334	-	-	-	18865.284	-	2A	yddK	-	-	-	14415.283	-	-	7
elaD	9277.656	-	-	-	-	-	3	yhcB	-	-	-	-	15683.094	-	7
rplA	-	-	-	9231.431	18862.577	-	3	yjjI	-	-	-	-	12871.398	-	7
rplK	9974.177	24235.603	-	-	-	-	3	yjjY	-	18872.48	-	-	-	-	7

Figure A-2-2. Quantitative sensitivity values for hypersensitive strains, as determined by colony density analysis software. Digital photographs of the colonies were analyzed quantitatively using density analysis software (higher value = more sensitive/less growth). A cut-off value of 7,000 was chosen so as to eliminate background noise. The values for the sites that are hypersensitive to one or more agent are listed alphabetically.

Sensitive Strains Found by Visual but not Quantitative Analysis				
Sensitive to BLM[a,b]		Sensitive to 5AZ[b]	Sensitive to ZEB[b]	Other strains[c]
fhuA	*ybeD*	*aceE*	*xapR*	*fur*
folB	*ybfJ*	*crr*	*xseA*	*pcnB*
JW5115	*ybgF*	*fepG*	*ycbK*	*recF*
JW5360	*ydcS*	*JW3133*		
metL	*yfiH*	*rhlB*		
pstS	*yidD*			
rpmF	*zapB*			
tolQ	*zwf*			

[a] Quantitative analysis was done on BLM 0.313 µg/ml plates; above strains only sensitive at BLM 0.5 µg/ml.

[b] Set quantitative analysis threshold high to avoid false positives. Some false negatives in weakly sensitive strains as a result.

[c] These strains were present on plates that were not analyzed by quantitative analysis.

Figure A-2-3. Inconsistencies between quantitative analysis and analysis by eye. Every site found via quantitative analysis was discovered by visual analysis. However, visual analysis discovered several sites weakly sensitive to different agents that were not discovered by quantitative analysis due to the following reasons: i) the strains were present on a plate that was not analyzed quantitatively, ii) quantitative analysis was done on a lower concentration than strains were sensitive to, iii) the quantitative analysis threshold was set high so as to avoid false positives, thus resulting in a few false negatives in weakly sensitive strains.

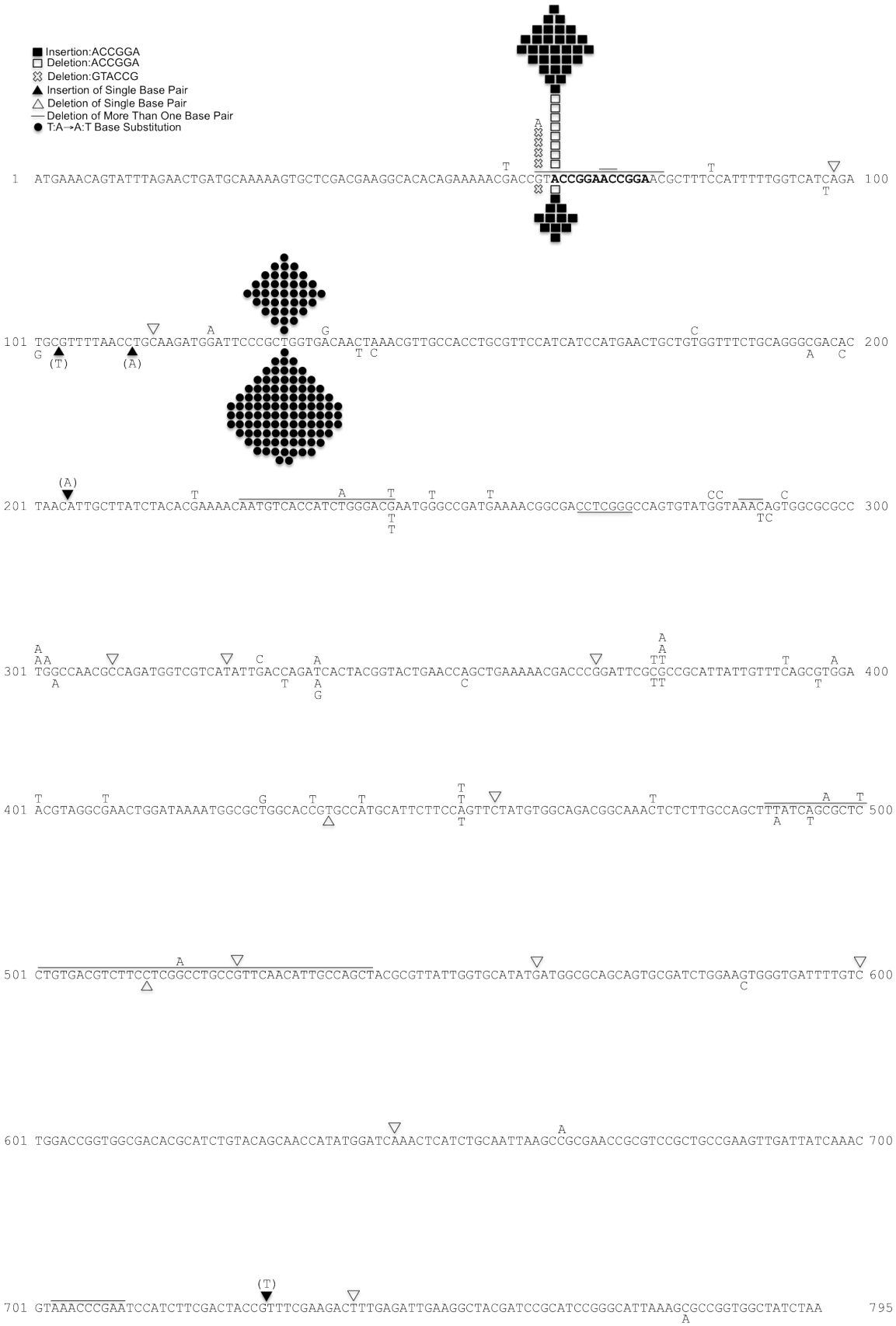
Insertion:ACCGGA
Deletion:ACCGGA
Deletion:GTACCG
Insertion of Single Base Pair
Deletion of Single Base Pair
Deletion of More Than One Base Pair
T:A→A:T Base Substitution
1 ATGAAACAGTATTTAGAACTGATGCAAAAAGTGCTCGACGAAGGCACACAGAAAAACGACCGTACCGGAACCGGAACGCTTTCCATTTTTGGTCATCAGA 100
101 TGCGTTTTAACCTGCAAGATGGATTCCCGCTGGTGACAACTAAACGTTGCCACCTGCGTTCCATCATCCATGAACTGCTGTGGTTTCTGCAGGGCGACAC 200
201 TAACATTGCTTATCTACACGAAAACAATGTCACCATCTGGGACGAATGGGCCGATGAAAACGGCGACCTCGGGCCAGTGTATGGTAAACAGTGGCGCGCC 300
301 TGGCCAACGCCAGATGGTCGTCATATTGACCAGATCACTACGGTACTGAACCAGCTGAAAAACGACCCGGATTCGCGCCGCATTATTGTTTCAGCGTGGA 400
401 ACGTAGGCGAACTGGATAAAATGGCGCTGGCACCGTGCCATGCATTCTTCCAGTTCTATGTGGCAGACGGCAAACTCTCTTGCCAGCTTTATCAGCGCTC 500
501 CTGTGACGTCTTCCTCGGCCTGCCGTTCAACATTGCCAGCTACGCGTTATTGGTGCATATGATGGCGCAGCAGTGCGATCTGGAAGTGGGTGATTTTGTC 600
601 TGGACCGGTGGCGACACGCATCTGTACAGCAACCATATGGATCAAACTCATCTGCAATTAAGCCGCGAACCGCGTCCGCTGCCGAAGTTGATTATCAAAC 700
701 GTAAACCCGAATCCATCTTCGACTACCGTTTCGAAGACTTTGAGATTGAAGGCTACGATCCGCATCCGGGCATTAAAGCGCCGGTGGCTATCTAA 795

Figure A-5-1. Diagram of mutations in *thyA* in wild-type and PNP-deficient strains. Wild-type and *pnp* mutants resistant to trimethoprim (TMP) were sequenced as described in Materials and Methods. This diagram depicts the spectrum of mutations types and locations in the *thyA* gene. Mutations depicted below the sequence are from wild-type TMP^r mutants, and mutations depicted above the sequences are from *pnp* TMP^r mutants. See text for explanation of results.