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Genetic Analysis of the 5-Azacytidine Sensitivity of *Escherichia coli* K-12

ASHOK S. BHAGWAT* AND RICHARD J. ROBERTS

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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DNA containing 5-azacytidine (5-azaC) has been shown to form stable detergent-resistant complexes with cytosine methylases. We reasoned that if 5-azaC treatment causes protein-DNA cross-links in vivo, then mutations in DNA repair and recombination genes may increase the sensitivity of a cell to 5-azaC. We found that although *recA* (defective) and *lexA* (induction-negative) mutants of *Escherichia coli* were very sensitive to the drug, mutations in *uvrA* and *ung* genes had little effect on cell lethality. The sensitivity of *recA* strains to 5-azaC was dose dependent and was enhanced by the overproduction of a DNA cytosine methylase in the cell. Unexpectedly, a strain of *E. coli* carrying a *recA* mutation and a deletion of the DNA cytosine methylase gene (*dcm*) was found to be significantly sensitive to 5-azaC. Study of mutations in the pyrimidine salvage pathway of *E. coli* suggests that direct phosphorylation of 5-azaC, rather than phosphorylation of its degradation products, is largely responsible for the lethal effects of the drug. The addition of uracil to the growth medium had little effect on cell lethality of *recA* mutants, but it partially reversed the inhibition of cell growth caused by 5-azaC. This reversal of the bacteriostatic effects of the drug could not be achieved by adding cytosine or orotic acid to the growth medium and required the presence of functional UMP-pyrophosphorylase (gene *upp*) in the cell.

5-Azacytidine (5-azaC), an analog of cytidine in which the C-H group at position 5 is replaced by a nitrogen, has a multitude of biological effects in a wide variety of organisms. These effects range from mutagenesis and inhibition of cell growth for bacteria to increase in sister chromatid exchange in hamster cells, as well as induction of differentiation in mouse cells and induction of synthesis of proteins such as hypoxanthine-guanine phosphoribosyl transferase and fetal globin in human cells. At the biochemical level, 5-azaC has been shown to cause hypomethylation of DNA, to produce defective rRNAs and tRNAs, and to inhibit protein synthesis (for recent reviews, see references 20–22, 38, and 39). The ability of 5-azaC to cause hypomethylation of DNA is believed to be the cause of many of the phenotypic effects of the drug (20–22).

The observation by Friedman (12) that a number of bacterial DNA cytosine methylases are inactivated by incubation with DNA containing 5-azaC was the first clue to the mechanism by which 5-azaC causes hypomethylation. On the basis of the results of that study and others, Santi et al. (34) suggested that 5-azaC acts as a mechanism-based inhibitor of cytosine methylases. Specifically, it was suggested that most cytosine methylases link covalently to position 6 of cytosine as a normal intermediate in their action. After the transfer of a methyl group from *S*-adenosylmethionine to position 5 of cytosine, the enzyme unlinks itself. 5-azaC, when incorporated into DNA or RNA at the site of methylation, sabotages this reaction by making the transfer of the methyl group to position 5 impossible. Therefore, cytosine methylases should form stable covalent complexes with DNA (or RNA) containing 5-azaC.

This prediction has been confirmed for the *Hpa*II methylase (35); for the *Eco*RII methylase, *Msp*I methylase, and Dcm methylase of *Escherichia coli* (14, 15); for the *Hha*I methylase (44); and for a DNA methylase from Friend erythroleukemia cells (5). Such methylase–5-azaC complexes are resistant to high salt, various detergents, and nucleases and can be trapped on nitrocellulose filters. Therefore, it seems likely that the reduction of cytosine methylase activity by 5-azaC and the subsequent hypomethylation of RNA and DNA in various organisms may be due to the function of covalent complexes between the methylases and RNA or DNA containing 5-azaC.

The presence of such complexes in vivo has not been demonstrated. If these complexes can exist stably in vivo, they would be expected to interfere with DNA replication and thus cause cell death. We reasoned that if such complexes were indeed lethal to cells, then mutants in DNA repair and recombination pathways may be more susceptible to 5-azaC than their wild-type parents are. The results of experiments performed to test this hypothesis and to identify the pathway by which *E. coli* processes 5-azaC are presented below. We also describe a method to separate the bacteriostatic effects of 5-azaC from its bactericidal effects on *E. coli*.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** Strains used in this study are listed in Table 1. pRecA430 is the plasmid Yrp12-*recA430* described by Keener et al. (23). It is a pBR322 derivative that carries the allele *430* of *recA*.

Test for 5-azaC sensitivity. The test for 5-azaC sensitivity was performed largely as described by Friedman (13). Cells were grown in M63 medium (36) supplemented with 0.1% Casamino Acids (Difco Laboratories, Detroit, Mich.), with the exception of JM109, which was grown in minimal medium without amino acid supplements. Strains TK365 and TK366 were grown in medium containing 2 μg of nicotinic acid per ml. When the cultures became turbid (about 5 to 30

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Genotype	Source
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169* *rpsL150*	F. Daldal
SE5000	MC4100 *recA56*	F. Daldal
AB1157	λ⁻ *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 rac hisG4 rfbD1 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	CGSC[a]
AB1886	AB1157 *uvrA6*	CGSC
AB2463	AB1157 *recA13*	CGSC
DM49	AB1157 *lexA3*	CGSC
GM30	*thr-1 ara-14 leuB6 fhuA31 lacY1 tsx-78 supE44 galK2 galE2 hisG4 rpsL xyl-5 thi-1 mtl-1*	M. G. Marinus
BH127	GM30 *recA56 srlC*::Tn*10*	This study
GM31	GM30 *dcm-6*	M. G. Marinus
BH128	GM31 *recA56 srlC*::Tn*10*	This study
RP4182	Δ(*supD-dcm-fla*) *trp gal rpsL*	J. S. Parkinson
BH112	RP4182 *recA56 srlC*	This study
W6	*relA1 spoT1 metB1*	CGSC
SO408	W6 *upp-11 rpsL254*	CGSC
BH117	SO408 *srlC*::Tn*10 recA56*	This study
SO415	SO408 *udk-2*	CGSC
BH121	SO415 *srlC*::Tn*10 recA56*	This study
SO441	SO408 *cdd-5*	CGSC
BH118	SO441 *srlC*::Tn*10 recA56*	This study
JM108	Δ(*lac-cod-pro*) *recA1 supE44 thi endA1 hsdR17 gyrA96 relA1*	J. Messing
JM109	JM108 F′ *traD36 proA⁺B⁺ cod⁺ lacI^qZΔM15*	P. Scolnik
TK365	*ara* Δ(*lac-pro*) *rpsL thi* $\phi 80_d$*lacZΔM15 supD zed-508*::Tn*10*	B. Duncan
TK366	TK365 *ung-1 nadB*	B. Duncan
JC10240	Hfr PO45 *recA56 srlC300*::Tn*10 thr-300 ilv-318 rpsE310 rel-1 thi-1*	M. G. Marinus
KM1187Δ21	F⁻ *uvrA* Δ*recA galK rpsL*	K. McEntee

[a] *E. coli* Genetic Stock Center.

Klett units), a small sample of the culture was removed, diluted twofold, and plated on LB plates (36). The remaining culture was divided into 2.5-ml aliquots, which were diluted twofold with the growth medium. 5-azaC was added to the cultures, and the cultures were incubated at 37°C with shaking for 30 min. After the 5-azaC treatment, cultures were centrifuged in an SS34 rotor (Sorvall Instruments, Wilmington, Del.) at 5,000 rpm for 5 min and the cell pellets were suspended in the same volume of LB medium. Appropriate dilutions of the cultures were plated on LB plates. When plasmids were present in the cells, appropriate antibiotics (ampicillin or tetracycline) were also added to the growth media and the plates.

The percent survival was defined as the number of CFUs per milliliter in the cultures after the 5-azaC treatment divided by the number of CFU per milliliter before the treatment and multiplied by 100. Duplicate or triplicate samples of each strain were treated with 5-azaC in each experiment, and the average percent survival was calculated. Whenever a mutant strain was tested for its 5-azaC sensitivity, the wild-type parent was tested in parallel. In all the experiments, one duplicate culture received no 5-azaC but was incubated at 37°C for 30 min along with the treated cultures. Dilutions from this untreated culture were also plated, and this served as a positive control for cell growth during the 30-min treatment period. Typically, this culture contained 30 to 70% more viable cells than did culture plated before the treatment period.

Construction of *recA* strains. The *recA56* allele was introduced into different strains in one of two ways. BH112, BH117, and BH118 were constructed by the interrupted mating of JC10240 with RP4182, SO408, and SO441, respectively. BH121, BH127, and BH128 were constructed by P1 transduction of the *recA56* allele to SO415, GM30, and GM31, respectively. In each case, sensitivities of the strain to UV light and mitomycin C (1 μg/ml) were used to indicate the presence of the defective *recA* allele. Resistances to 5-fluorouracil (2.5 μg/ml), 5-fluorouridine (10 μg/ml), 5-fluorodeoxycytidine (10 μg/ml), and 5-fluorocytosine (10 μg/ml) were used to test for the *upp*, *udk*, *cdd*, and *cod* mutations, as described by Hammer-Jespersen and Munch-Petersen (17). The absence of the *dcm* gene in BH112 was confirmed by introducing pBR322 into the strain, extracting the plasmid from the cells, and cutting the DNA with *Eco*RII. The susceptibility of this DNA to *Eco*RII confirmed that the Δ*dcm* genotype of RP4182 was intact in BH112.

Growth of cultures in the presence of normal pyrimidines. Cells were grown overnight in M63 supplemented with 0.1% Casamino Acids and the appropriate pyrimidine. Fresh cultures were started by inoculating 10 ml of the growth medium (containing the same pyrimidine) with 50 μl of the overnight culture. After 1 h of shaking at 37°C, cultures were divided in half and 5-azaC was added to one half of the culture at a concentration of 20 μg/ml. Cultures were further incubated at 37°C with shaking, and the cell densities were monitored with a Klettmeter (Klett Manufacturing Co., Inc., New York, N.Y.).

RESULTS

***recA* mutants were sensitive to 5-azaC.** *E. coli* K-12 carries a gene designated *dcm* that codes for a DNA cytosine methylase (28). Dcm is the only known DNA cytosine methylase in *E. coli* K-12, and it methylates position 5 of the second cytosine within the sequence 5′-CC(A/T)GG-3′ (28, 29). Friedman (14) has shown that the Dcm protein forms covalent complexes with 5-azacytosine-containing DNA in vitro. It occurred to us that treating *E. coli* with 5-azaC was likely to create such protein-DNA complexes in vivo and that these complexes were likely to interfere with essential cellular functions. Since the *dcm*⁺ strains used by Friedman (13) are fairly resistant to the drug, it appeared that there may be a repair system in *E. coli* that removes this DNA damage.

The principal DNA repair systems in *E. coli* are controlled by the gene *recA*. A variety of different types of damage to the DNA in the cell results in the activation of the RecA protein, which in turn induces a number of genes involved in the repair of the damage (for reviews, see references 41 and 42). To determine the role played by *recA* in the repair of DNA damage caused by 5-azaC, we tested several *dcm*⁺ strains carrying defective *recA* alleles and their *recA*⁺ parents for sensitivity to 5-azaC. The *recA* mutant alleles were defective in both homologous recombination and induction of DNA repair genes. The results of two such pairs are presented in Table 2. The *recA*⁺ parents were only slightly sensitive to treatment with 20 μg of 5-azaC per ml, whereas their *recA* derivatives were approximately 1,000-fold more sensitive. Similar results have been obtained with other defective *recA* alleles as well as with a *recA* deletion strain (data not shown). It is interesting that 5-azaC does not resemble other base analogs such as 2-aminopurine and

TABLE 2. Sensitivity of *recA* strains to 5-azaC

Strain	Relevant genotype	5-azaC concn (μg/ml)	CFU/ml[a]	% Survival
MC4100	*dcm*$^+$ *recA*$^+$	0	2.7×10^7	100
		20	$(1.9 \pm 0.43) \times 10^7$	71.0 ± 16.0
SE5000	*dcm*$^+$ *recA56*	0	4.3×10^7	100
		20	$(1.9 \pm 0.60) \times 10^4$	0.045 ± 0.01
AB1157	*dcm*$^+$ *recA*$^+$	0	7.3×10^7	100
		20	$(2.04 \pm 0.08) \times 10^7$	27.9 ± 1.0
AB2463	*dcm*$^+$ *recA13*	0	3.4×10^6	100
		20	$(1.67 \pm 0.58) \times 10^3$	0.049 ± 0.017
SE5000	*dcm*$^+$ *recA56*	0	8.9×10^7	100
		0.02	8.2×10^7	91
		0.20	1.9×10^7	21
		2.0	1.7×10^5	0.19
		20.0	4.6×10^4	0.051
		100.0	2.4×10^4	0.027

[a] On LB plates.

5-bromouracil in this respect. Unlike 5-azaC, 2-aminopurine and 5-bromouracil do not efficiently kill *recA* mutants (32, 43). The relative insensitivity of *dcm*$^+$ *recA*$^+$ strains to 5-azaC has been noted previously by Friedman (13).

This sensitivity of *recA* mutants is dependent on the concentration of 5-azaC used. The percent survival of SE5000, a *dcm*$^+$ *recA* strain, ranged from 91 to 0.027%, respectively, when the 5-azaC concentration was increased from 0.02 to 100 μg/ml (Table 2). When cells were treated with 20 μg of 5-azaC per ml for 30 min, as described in Materials and Methods, the survival of most *recA* strains was reduced to between 0.2 and 0.02% of that of the untreated controls.

A majority of the cells that survive the 5-azaC treatment may not be significantly more resistant to a second treatment of the drug. Eight colonies of strain SE5000 that survived a 30-min treatment of 100 μg of 5-azaC per ml were picked, grown, and tested with 20 μg of the drug per ml. Only one of these clones was significantly more resistant to the drug than the original SE5000 (data not shown). As judged by its UV sensitivity, the resistant clone appeared not to have lost the *recA* mutation. It is possible that this clone may have a defect in the transport or the processing of 5-azaC (see below).

Overproduction of the Dcm protein increased sensitivity to 5-azaC. If the lethality caused by 5-azaC is due to the formation of covalent complexes between 5-azaC in the DNA and a cytosine methylase, increasing the amount of methylase in the cell should increase the level of 5-azaC sensitivity. This was found to be the case. Plasmid pDCM1 is a pBR322 derivative carrying the *dcm* gene of *E. coli*. It produces about 30-fold as much Dcm protein as the chromosomal *dcm* gene (4). Whereas most *recA*$^+$ strains carrying the *dcm*$^+$ gene on the chromosome were only slightly sensitive to 5-azaC (Tables 2 and 3), RP4182 (Δ*dcm recA*$^+$) carrying pDCM1 was very sensitive to the drug (Table 3). The sensitivity of the *recA* mutant SE5000 to 5-azaC was increased about fivefold by the introduction of pDCM1 into the cells (Table 3). This increase in sensitivity, although much smaller than the 1,000-fold increase in sensitivity for RP4182, was reproducible. This may mean that in a *recA* mutant background some repair of potentially lethal damage caused by 5-azaC does take place but that overproduction of the cytosine methylase overwhelms the repair capabilities. A correlation between sensitivity to 5-azaC and the amount of deoxycytosine methylase in the cell for *recA*$^+$ strains has been noted by Friedman (13). The results presented here suggest that potentially lethal DNA damage caused by 5-azaC in *E. coli* increases with increasing amounts of deoxycytosine methylase in the cells, regardless of the state of DNA repair in the cells.

Effect of other DNA repair mutations on 5-azaC sensitivity of *E. coli*. The LexA protein is a repressor of *recA* and other DNA repair-related genes. The key step in the induction of repair genes in *E. coli* is the proteolytic cleavage of LexA by the activated RecA (for reviews, see references 27, 41, and 42). The *lexA3* mutation leads to the synthesis of a LexA protein that is resistant to proteolytic cleavage and thus prevents the induction of DNA repair genes in the cell (26). To test whether the sensitivity of *recA* (defective) mutants to 5-azaC is due to the lack of induction of a gene(s) involved in DNA repair, the cells carrying the *lexA3* mutation were

TABLE 3. 5-azaC sensitivity of strains carrying pDCM1[a]

Strain	Relevant genotype	Plasmid	CFU/ml[b]		% Survival
			Untreated	Treated[c]	
RP4182	Δ*dcm recA*$^+$	pBR322	3.8×10^7	$(4.6 \pm 0.90) \times 10^7$	121 ± 24
		pDCM1	2.5×10^7	$(3.3 \pm 0.06) \times 10^4$	0.13 ± 0.002
SE5000	*dcm*$^+$ *recA56*	pBR322	2.4×10^7	$(2.4 \pm 0.52) \times 10^4$	0.10 ± 0.02
		pDCM1	2.7×10^7	$(6.0 \pm 0.85) \times 10^3$	0.022 ± 0.003

[a] pDCM1 is a pBR322 derivative that carries the *dcm*$^+$ gene and produces 30-fold as much Dcm protein as the chromosomal *dcm*$^+$ gene does.
[b] On LB plus ampicillin plates.
[c] 5-azaC was used at 20 μg/ml.

TABLE 4. Sensitivity of DNA repair mutants to 5-azaC

Strain	Relevant genotype	Plasmid	CFU/ml[a]		% Survival
			Untreated	Treated[b]	
AB1157	*uvrA*$^+$		4.8×10^7	$(1.6 \pm 0.08) \times 10^7$	32.3 ± 1.7
AB1886	*uvrA6*		6.4×10^7	$(1.9 \pm 0.11) \times 10^7$	30.2 ± 1.8
TK365	*ung*$^+$		5.1×10^7	2.5×10^7	48
TK366	*ung-1*		4.5×10^7	2.2×10^7	50
AB1157	*lexA*$^+$		1.5×10^7	$(1.3 \pm 0.15) \times 10^7$	86.6 ± 1.0
DM49	*lexA3*		6.6×10^7	$(4.6 \pm 2.4) \times 10^4$	0.069 ± 0.037
KM1187Δ21	Δ*recA*		2.9×10^7	$(2.7 \pm 0.39) \times 10^5$	0.93 ± 0.14
		pRecA$^+$	1.5×10^7	$(4.3 \pm 1.2) \times 10^6$	29.5 ± 8.5
		pRecA430	1.3×10^7	$(2.4 \pm 0.28) \times 10^6$	18.7 ± 2.2

[a] On LB or LB plus tetracycline plates.
[b] 5-azaC was used at 20 μg/ml.

assayed for sensitivity to 5-azaC. It was found that a strain carrying the *lexA3* mutation was over 1,000-fold more sensitive than its *lexA*$^+$ parent was (Table 4).

One of the genes induced by the *recA-lexA* system after DNA damage (during the so-called SOS response) is *uvrA* (24). It is involved in excision repair of pyrimidine dimers and other bulky DNA damage products (for a review, see reference 42). Because the methylase complexed with DNA is a bulky adduct on the DNA, we tested whether a functional *uvrA* was essential for resistance to 5-azaC. We found that a *uvrA* mutant was only as sensitive to 5-azaC as its *uvr*$^+$ parent was (Table 4). We also found that a *recA uvrA* mutant was sensitive to 5-azaC to roughly the same extent as its *recA uvrA*$^+$ parent was (data not shown). Therefore, the *uvr*-dependent excision repair pathway cannot be responsible for the resistance of *recA*$^+$ strains to 5-azaC.

The *recA* gene is also involved in a DNA repair pathway that does not involve *uvrA* and most other SOS-induced genes, but depends on the ability of the RecA protein to mediate homologous recombination (for a review, see reference 18). To evaluate the relative importance of the two functions of the RecA protein in the resistance of wild-type *E. coli* to 5-azaC, we studied the *recA430* mutation. This mutation substantially reduces the ability of *recA* to induce the SOS response but leaves the recombinational ability of the protein largely intact (30). Introduction of a plasmid carrying the *recA430* allele into a Δ*recA* cell rendered the cell fairly insensitive to 5-azaC (Table 4). A strain carrying the *recA430* mutation in the chromosome was also insensitive to 5-azaC (data not shown). Thus, although the sensitivity of the *lexA3* mutation to 5-azaC suggests that some SOS response is essential for the cell to survive the damage caused by the drug, the small amount of SOS induction seen in the *recA430* background (9, 10) was sufficient for this purpose. Since the *recA* gene is among the many genes normally repressed by *lexA*, it is possible that the requirement for some SOS response in the cell for its resistance to 5-azaC is related to the overproduction of RecA rather than to the activation of RecA. This hypothesis is supported by our recent studies with an operator-constitutive (o^c) mutant of *recA*. A *recA* (o^c) *lexA3* double mutant was found to be significantly less sensitive to 5-azaC than its *recA*$^+$ parent was (data not shown). Finally, preliminary studies also indicate that some function of *recA* was required for the repair of 5-azaC-caused damage, even in a cell that constitutively expressed SOS genes due to a defective *lexA*. A *recA* (defective) *lexA* (defective) strain was considerably more sensitive to 5-azaC then its *recA*$^+$ parent was (data not shown).

Processing of 5-azaC in *E. coli*. In a number of different organisms, 5-azaC is processed by the pyrimidine salvage pathway and incorporated into both RNA and DNA. Specifically, it is phosphorylated by uridine-cytidine kinase and deaminated to 5-azauridine by cytidine deaminase (for a review, see reference 40). The expected products of the processing of 5-azaC in *E. coli* and the enzymes involved are shown in Fig. 1. The presence of some of these products after incubation of 5-azaC with *E. coli* cell extracts has been reported previously (6).

Cytidine deaminase of *E. coli* is thought to convert 5-azaC to 5-azauridine (8). In solution, 5-azaC has been shown to decompose into 5-azacytosine, 5-azauracil, and other products depending on pH (31). Therefore, it is possible that the lethal effects of 5-azaC on *recA* cells are due to its incorporation into DNA as 5-azauracil. We reasoned that if the potentially lethal products(s) of 5-azaC in *E. coli* arises by a unique enzymatic pathway, then mutations that inactivate one or more enzymes in the pathway should make *recA* mutant cells insensitive to 5-azaC. Thus, we constructed *recA upp*, *recA cdd*, and *recA udk* strains and tested them for sensitivity to 5-azaC. We also tested a prexisting *recA* Δ*cod* strain for sensitivity to the drug.

The *recA56* derivatives of *upp* and *upp cdd* mutants were, respectively, 1,000- and 10,000-fold more sensitive to 5-azaC than their *recA*$^+$ parents were (Table 5). Therefore, the conversion of 5-azaC by cytidine deaminase (Fig. 1, gene *cdd*) to 5-azauridine or the conversion of 5-azauracil in the cell to 5-azauracil-5′-monophosphate by UMP-pyrophosphorylase (Fig. 1, gene *upp*) was not essential for the lethal effects of the drug. Similarly, the Δ*cod recA* mutant was roughly as sensitive to 5-azaC as was a Δ*cod recA* carrying a functional *cod* on an F′ (Table 5). Thus, the conversion of 5-azaC to a lethal form did not require the synthesis or the processing of either 5-azauridine or 5-azauracil. In contrast to these mutants, the *recA56* derivative of the *upp udk* mutant was as insensitive to the drug as the *recA*$^+$ parent was (Table 5). Therefore, conversion of 5-azaC to 5-azaC-5′-monophosphate (5-aza CMP) led to a potentially lethal form of the drug. Since the only deoxynucleoside triphosphate that is likely to be derived from 5-aza CMP by the salvage pathway is 5-aza-dCTP (Fig. 1), the lethal form of the drug in the DNA is likely to be 5-azacytosine.

When uracil instead of thymine is incorporated into *E. coli* DNA, it is removed by a pathway that requires the enzyme

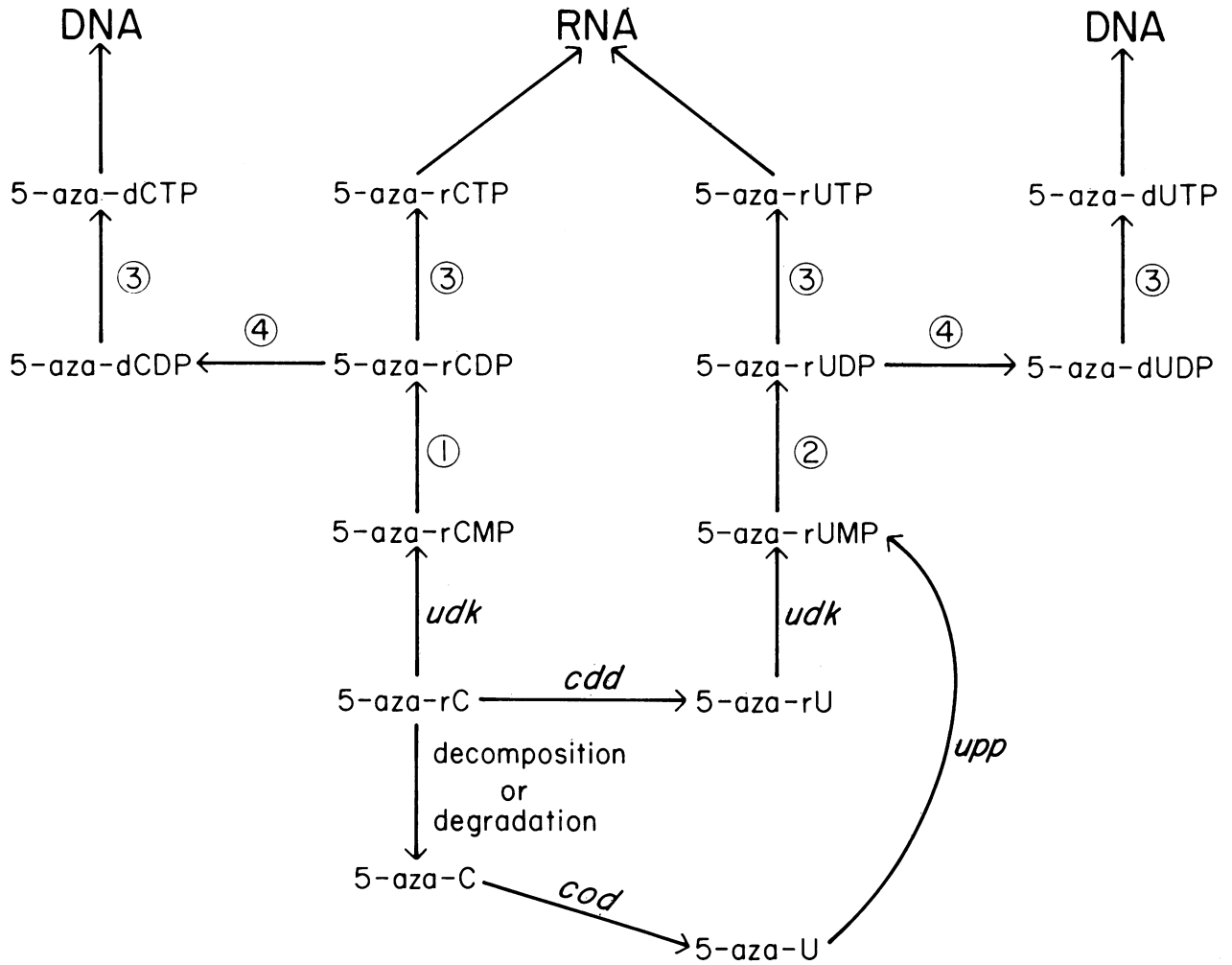

FIG. 1. Processing of 5-azaC by *E. coli*. The pathway by which *E. coli* utilizes exogeneous cytidine and uridine is described. Mutants in the genes that were tested for 5-azaC sensitivity are indicated by three letter codes. Other enzymes in the pyrimidine salvage pathway are identified by numbers 1 through 4. *udk*, Uridine-cytidine kinase; *cdd*, cytidine deaminase; *cod*, cytosine deaminase; *upp*, UMP-pyrophosphorylase; 1, cytidylate kinase; 2, uridylate kinase; 3, nucleoside diphosphokinase; 4, ribonucleotide reductase; C, cytosine; U, uracil. Prefixes r- and d- indicate ribo- and deoxyribo- forms of nucleosides, respectively. Thus, 5-azaC is 5-aza-rC.

uracil-*N*-glycosylase (gene *ung*; 25). If the potentially lethal form of 5-azaC in the DNA is 5-azauracil, then cells defective in the removal of uracil from DNA may be more sensitive to 5-azaC than normal cells are. This was found not to be the case. A strain with a mutation in the *ung* gene was no more sensitive to 5-azaC than its *ung*$^+$ parent was (Table 4). Hence, uracil removal by uracil-*N*-glycosylase could not be responsible for the resistance of wild-type *E. coli* to 5-azaC.

Ability of nucleosides and bases to reverse lethal effects of 5-azaC. When uridine or cytidine was added to the growth medium along with 5-azaC, the lethal effects of 5-azaC were suppressed in a concentration-dependent manner (Table 6). At the highest concentration of the normal nucleosides tested (100 μg/ml), the cultures contained more viable cells at the end of the 30-min treatment period than at the start, suggesting a reversal of growth inhibition as well as a suppression of lethality. In contrast, neither thymidine nor uracil was able to reverse the lethal effects of 5-azaC completely, even at the highest concentration used (Table 6, 100 μg/ml).

These results are consistent with the conclusion drawn above that the uridine-cytidine kinase is principally responsible for the conversion of 5-azaC to 5-aza CMP and that the lethal effects of the 5-azaC are mainly due to the subsequent processing of this monophosphate derivative. Normal cytidine and uridine can be expected to compete successfully with 5-azaC for phosphorylation, whereas thymidine and uracil are not expected to do so. Similarly, if the lethal effects of 5-azaC were due to its conversion to 5-azauridine and its subsequent incorporation into DNA as 5-aza-dUTP, thymidine should have been able to reverse this effect. This was not the case (Table 6). The moderate reduction in lethality caused by 5-azaC at the highest concentration of thymidine may be due to a general suppression of the pyrimidine salvage pathway. 5-azaC is known to decompose into 5-azacytosine, which may be converted to 5-azauracil by cytosine deaminase. The inability of uracil to reverse the lethal effects of 5-azaC (Table 6) again suggests that the degradation or decomposition products of 5-azaC are not responsible for its lethal effects.

Uracil can partially reverse the bacteriostatic effects of 5-azaC. 5-azaC causes reversible growth inhibition in many bacterial strains, including *E. coli* (13, 19). We found that

TABLE 5. Sensitivity of mutants in the pyrimidine salvage pathway to 5-azaC

Strain	Relevant genotype	CFU/ml[a]		% Survival
		Untreated	Treated[b]	
SO408	*upp*	3.2×10^7	$(4.1 \pm 0.43) \times 10^7$	131 ± 13.7
BH117	*upp recA56*	1.9×10^7	$(3.6 \pm 0.13) \times 10^4$	0.19 ± 0.07
SO441	*upp cdd*	6.9×10^7	$(7.6 \pm 1.9) \times 10^7$	110 ± 27.1
BH118	*upp cdd recA56*	2.9×10^7	$(8.3 \pm 3.5) \times 10^3$	0.012 ± 0.005
JM108	*Δcod recA1*	5.0×10^6	$(1.20 \pm 0.46) \times 10^3$	0.024 ± 0.0092
JM109	*Δcod recA1/F' cod*$^+$	7.7×10^7	$(1.23 \pm 0.28) \times 10^5$	0.16 ± 0.037
SO415	*upp udk*	2.1×10^7	$(1.2 \pm 0.16) \times 10^7$	57.9 ± 7.8
BH121	*upp udk recA56*	1.8×10^7	$(1.2 \pm 0.30) \times 10^7$	69.8 ± 17.1

[a] On LB plates
[b] 5-azaC was used at 20 μg/ml.

although uracil was unable to reverse the bactericidal effects of 5-azaC, it was largely able to reverse the bacteriostatic effects. This can be seen in Fig. 2A. RP4182, a *Δdcm recA*$^+$ strain, was not significantly killed by 5-azaC (Table 3), but it grew extremely poorly in the presence of 5-azaC. The growth rate of the treated culture was 4.5% of that of the untreated culture. When uracil was present in the growth medium at 1 μg/ml, little change in the growth inhibition was seen (Fig. 2A). At 10 μg of uracil per ml the growth rate was restored to 39.5% of that of the control, but the culture did not reach the same stationary-phase cell density as the untreated control did (Fig. 2A). Increasing the uracil concentration to 100 μg/ml restored the growth rate to 64.9% of the control value, and the stationary-phase densities of the drug-treated and untreated cultures were nearly the same (Fig. 2A). Increasing the uracil concentration further did not increase the growth rate beyond 75% of the control value (data not shown). This reversal of growth inhibition was specific for uracil. Other pyrimidines, such as cytosine or orotic acid, were inefficient at reversing the growth (Fig. 2B). The modest reversal of growth inhibition seen with cytosine may be due to its conversion to uracil by the cytosine deaminase in the cell (Fig. 1).

A significant alleviation of the bacteriostatic effect of 5-azaC by uracil could also be seen for *E. coli* strains that are *dcm*$^+$. Uracil at a concentration of 100 μg/ml restored the growth rates of several such strains treated with 5-azaC to between 30 and 65% of the control values (Fig. 3 and data not shown). For instance, in the presence of 5-azaC and the absence of uracil, MC4100 (*dcm*$^+$ *recA*$^+$) grew well for about 2 h, but then its growth rate dropped to 7.2% of that of the untreated culture. However, when uracil was also present in the medium, the growth rate increased to 36% of that of the untreated control (Fig. 3). SE5000, a *recA* derivative of MC4100, also grew poorly in the presence of 5-azaC. Its growth rate dropped to 8.7% of the control value (data not shown). However, 5-azaC was lethal to a *recA* strain in addition to being bacteriostatic (Table 2). Clearly, growth inhibition or a reversal of growth inhibition cannot be observed in dead cells. Hence, uracil was unable to significantly reverse the growth inhibition of SE5000 caused by 5-azaC. The addition of uracil to the 5-azaC-treated culture of SE5000 increased its growth rate from 8.7 to only 11.7% of that of the control culture (data not shown).

It should be noted that a functional *upp* gene must be present in the cell if uracil is to reverse the bacteriostatic effect of 5-azaC. Whereas the growth of SO408, a *upp* mutant strain, was inhibited by 5-azaC regardless of the presence of uracil in the growth medium (Fig. 4), this was not the case for its *upp*$^+$ parent W6. The parent strain was inhibited by 5-azaC, but this inhibition could be largely eliminated by adding uracil to the growth medium (Fig. 4).

***dcm recA* mutants were sensitive to 5-azaC.** As noted earlier, most *recA*$^+$ *dcm*$^+$ strains were fairly insensitive to 5-azaC. This could also be seen for GM30 (Table 7). The *dcm* mutant derivative of the strain (GM31) was sensitive to the drug at about the same level (Table 7). Since GM30 was only marginally sensitive to 5-azaC, it is difficult to draw firm conclusions about the effect of the *dcm* mutation on 5-azaC sensitivity based on the sensitivities of GM30 and GM31. Thus, we investigated the sensitivity to 5-azaC of two *dcm recA* double mutants. If Dcm is the only deoxycytosine methylase in *E. coli* K-12 and if the lethal effects of 5-azaC are due entirely to the formation of covalent links between 5-azaC incorporated into DNA and a cytosine methylase, *dcm* mutants should be insensitive to 5-azaC regardless of the state of *recA* in the cells. This was found not to be the case.

Both *dcm recA* and *Δdcm recA* strains were sensitive to

TABLE 6. Effect of normal pyrimidines on the 5-azaC sensitivity of a *recA* strain[a]

Pyrimidine	Pyrimidine concn (μg/ml)	5-azaC concn (μg/ml)	CFU/ml[b]	% Survival
None	0	0	3.2×10^7	100
None	0	20	4.0×10^4	0.13
Uridine	1	20	1.4×10^5	0.43
	100	20	3.8×10^7	119
Cytidine	1	20	3.6×10^5	1.1
	100	20	4.4×10^7	138
None	0	0	2.5×10^7	100
None	0	20	2.7×10^4	0.11
Thymidine	1	20	6.9×10^4	0.28
	5	20	7.5×10^4	0.30
	25	20	2.4×10^5	0.97
	100	20	7.3×10^5	2.9
None	0	0	4.3×10^7	100
None	0	20	$(6.67 \pm 3.06) \times 10^3$	0.016 ± 0.007
Uracil	10	0	4.6×10^7	100
	10	20	$(1.18 \pm 0.11) \times 10^5$	0.26 ± 0.025
	100	0	4.3×10^7	100
	100	20	$(1.33 \pm 0.13) \times 10^5$	0.31 ± 0.03

[a] SE5000, *dcm*$^+$ *recA56*.
[b] On LB plates.

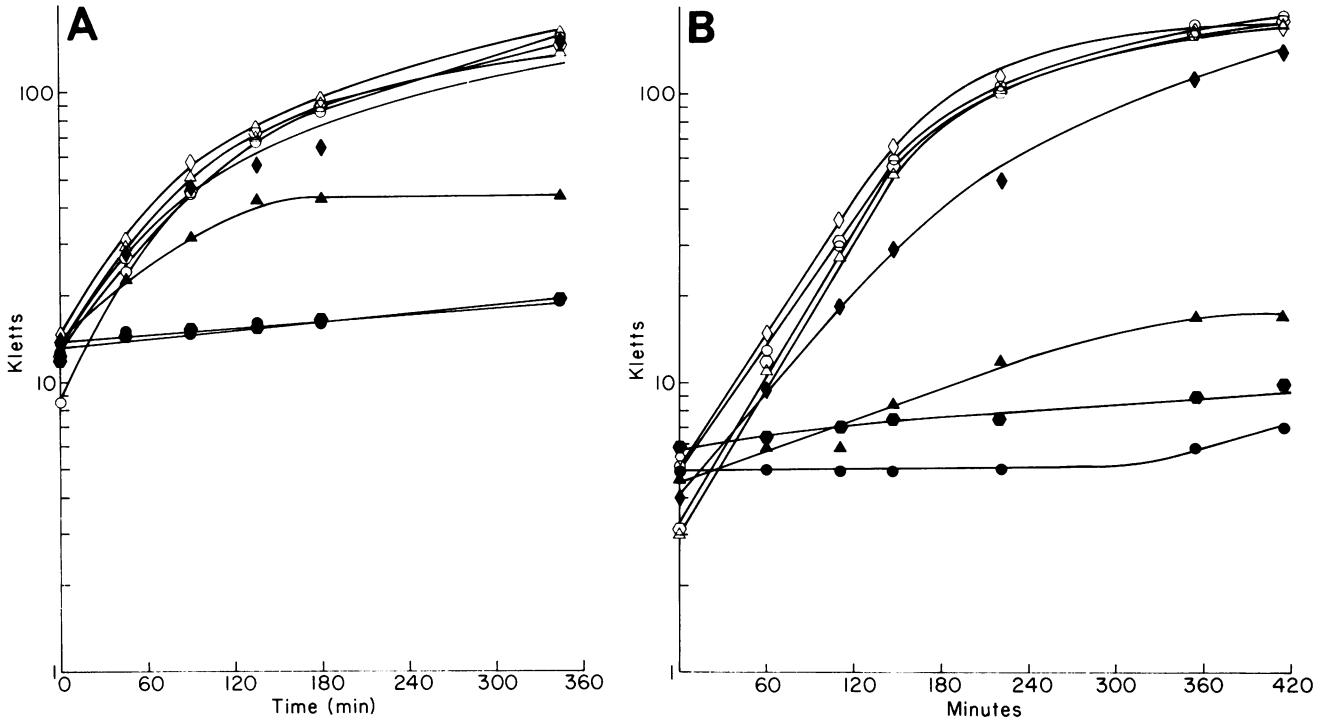

FIG. 2. (A) Effect of uracil on growth inhibition of a Δ*dcm* strain caused by 5-azaC. Fresh cultures of RP4182 containing various amounts of uracil were started by diluting overnight cultures with the same amounts of uracil. After 1 h of growth, the cultures were divided in half and 5-azaC was added to one of the two cultures to a concentration of 20 μg/ml. Cell densities of the cultures were monitored over several hours. Symbols: ○, no uracil and no 5-azaC; ●, no uracil plus 5-azaC; ⬡, 1 μg of uracil per ml and no 5-azaC; ⬢, 1 μg of uracil per ml plus 5-azaC; △, 10 μg of uracil per ml plus 5-azaC; ▲, 10 μg of uracil per ml plus 5-azaC; ◇, 100 μg of uracil per ml and no 5-azaC; ◆, 100 μg of uracil per ml plus 5-azaC. (B) Effects of cytosine and orotic acid on the growth inhibition of a Δ*dcm* strain caused by 5-azaC. From four overnight cultures of RP4182 containing, respectively, no added pyrimidines, 1 mM uracil, 1 mM cytosine, or 1 mM orotic acid, fresh cultures were started. After 1 h of growth, the cultures were divided in two and 5-azaC was added to one half to a concentration of 20 μg/ml. Cell densities were monitored over several hours. Symbols: ○, no uracil and no 5-azaC; ●, no uracil plus 5-azaC; ◇, uracil and no 5-azaC; ◆, uracil plus 5-azaC; △, cytosine and no 5-azaC; ▲, cytosine plus 5-azaC; ⬡, orotic acid and no 5-azaC; ⬢, orotic acid plus 5-azaC.

5-azaC (Table 7). Although BH128 (*dcm recA*) was less sensitive to 5-azaC than BH127 (*dcm*$^+$ *recA*) was, it was more sensitive to the drug than its *recA*$^+$ parent GM31 was (Table 7). Similarly, the survival of BH112 (Δ*dcm recA*) after 5-azaC treatment was reproducibly 25-fold lower than the survival of its *recA*$^+$ parent RP4182. It is unclear why BH112 was more sensitive to the drug than BH128 was. Since the *dcm* mutation within GM31 was obtained by chemical mutagenesis (28), it can be argued that the sensitivity of BH128 to 5-azaC is due to the leakiness of the *dcm* mutation. The same cannot be argued for RP4182. Genetic data as well as the results of Southern blotting with the cloned *dcm* gene as a probe suggest that this strain is completely missing the *dcm* gene (2, 4). Thus, these data suggest either that there are additional types of lethal damage caused by 5-azaC or that there is at least one additional DNA cytosine methylase in *E. coli*.

DISCUSSION

We have presented here the results of a genetic study of the effects of 5-azaC on *E. coli* K-12. Several interesting points have emerged from this study. (i) The dependence of cell survival on the activity of some DNA repair genes but not others strongly indicates that the damage caused by 5-azaC is repaired in *E. coli* and that the pathway for this repair may be specific for such damage. If the damage concerned is methylase-DNA cross-links, as is likely the case, the repair pathway may be involved in the repair of other protein-DNA cross-links in *E. coli*, as well. (ii) Several lines of evidence presented here suggest that phosphorylation of 5-azaC and not of its decomposition or degradation products is responsible for the lethal damage. (iii) We showed that the bacteriostatic effects of 5-azaC can be partially overcome without significantly altering the bactericidal effects arising from DNA damage. (iv) The study of a Δ*dcm recA* mutant suggests either that *E. coli* carries an unknown DNA cytosine methylase(s) or that 5-azaC causes lethal damage in additional ways not presently understood.

The limited survey of sensitivities of DNA repair mutants to 5-azaC presented here suggests that the damage caused by 5-azaC in the cell may be repaired by the postreplication repair pathway. Postreplication repair (also known as daughter-strand gap repair) cannot take place in *recA* (defective) or *lexA* (induction-negative) genetic backgrounds, but it is not dependent upon the *uvr* genes (16, 33). In addition, the ability of the RecA protein to mediate homologous recombination appears to be more important in this kind of repair than is its ability to induce the SOS response (45). Therefore, our data suggest that cells in which postreplication repair can take place are quite resistant to 5-azaC, whereas cells that are defective in this kind of repair are sensitive to the drug (Tables 2 and 4). However, a biochemical analysis of the events following 5-azaC treatment in different DNA repair

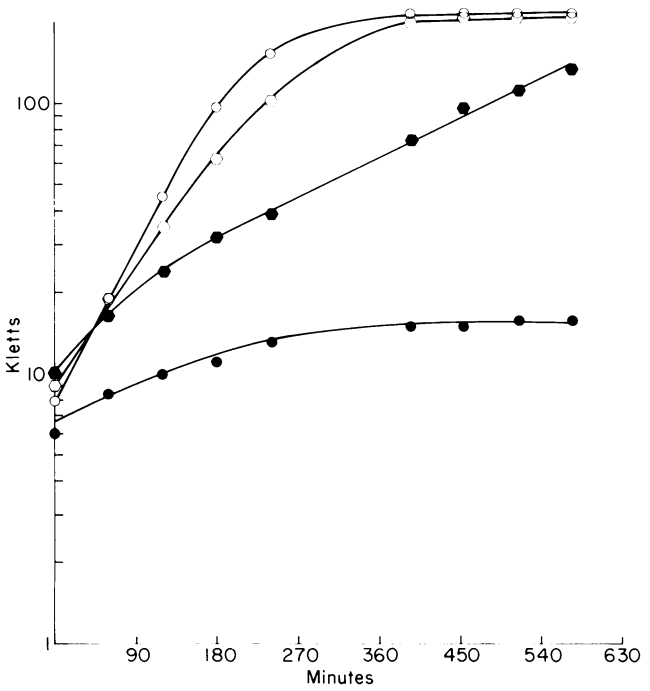

FIG. 3. Effect of uracil on growth inhibition of a *dcm*$^+$ strain. Two cultures of MC4100 were prepared. One contained 100 μg of uracil per ml. From these overnight cultures, fresh cultures were started. After 1 h of growth, the cultures were divided in two. To one half, 5-azaC was added to 20 μg/ml, and the cell densities of the cultures were monitored over several hours. Symbols: ○, no uracil and no 5-azaC; ●, no uracil plus 5-azaC; ⬡, uracil and no 5-azaC; ⬢, uracil plus 5-azaC.

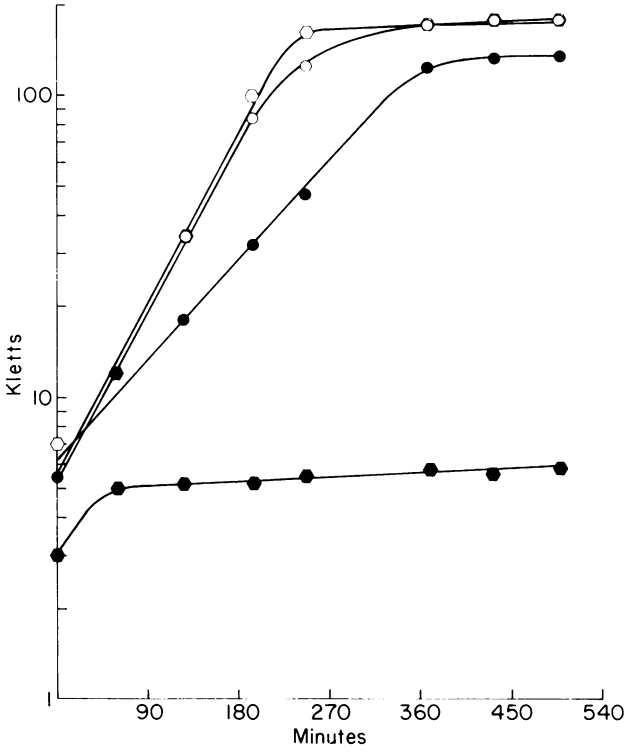

FIG. 4. Effect of uracil on the growth inhibition of a *upp* mutant strain. Strains W6 (*upp*$^+$) and SO408 (*upp* mutant) were grown with 100 μg of uracil per ml. From these overnight cultures, fresh cultures were started. After 1 h of growth, the cultures were divided in two. 5-azaC was added to one half, and the cell densities were monitored over several hours. Symbols: ○, W6 with no 5-azaC; ●, W6 with 5-azaC; ⬡, SO408 with no 5-azaC; ⬢, SO408 with 5-azaC.

mutants is needed before a firm statement about a repair pathway can be made.

During the preparation of this article, Barbe et al. (3) published a study of 5-azaC that bears on some of the conclusions drawn above. In a manner similar to ours, these investigators have concluded that the *recA* mutants of *E. coli* are sensitive to 5-azaC. These authors have also presented data that suggest that strains GM31, GM48, GM41, and MC1061 are slightly (1.5- to 5-fold) less sensitive to 5-azaC than AB1157 is. On the basis of these data, the authors concluded that mutations in *dcm*, *dam*, and *hsdR* genes lead to increased resistance to 5-azaC. This is difficult to understand for two reasons. (i) Without knowing the standard deviation within the data, a small effect such as this cannot be adequately evaluated. (ii) Since none of the four mutant strains is closely related to AB1157, the difference in 5-azaC sensitivities may simply be a reflection of the rather different genetic histories of these strains. In fact, we observed that AB1157 was significantly more sensitive to 5-azaC than most other *recA*$^+$ strains were (Table 2 and data not shown).

Our observation that BH112, a Δ*dcm recA* strain, was quite sensitive to 5-azaC (Table 7) is difficult to explain on the basis of present knowledge about cytosine methylation in *E. coli* and the in vitro studies about interaction between cytosine methylases and DNA containing 5-azaC. The only known DNA cytosine methylase gene in *E. coli* K-12, *dcm*, is completely deleted from BH112 (4). Thus, if the potentially lethal damage caused by 5-azaC involves cytosine methylase–5-azacytosine complexes, then such damage should be absent in BH112 treated with 5-azaC. It is possible that *E. coli* carries an undiscovered DNA cytosine methylase(s) with a sequence specificity different from that of *dcm* or that it contains other proteins that interact with cytosine in DNA in a manner similar to that of the cytosine methylases. Alternatively, 5-azaC or a derivative of it may cause potentially lethal DNA damage in a manner not appreciated before.

Data presented here suggest that the bactericidal and the bacteriostatic effects of 5-azaC may be caused by different final products of the drug. The lethal effects of the drug require its conversion to 5-aza CMP, which is most likely incorporated into DNA as 5-azacytosine. This is consistent with our observation that the overproduction of a cytosine methylase in the cell increases its sensitivity to 5-azaC

TABLE 7. Sensitivity of *dcm* mutants to 5-azaC

Strain	Relevant genotype	CFU/ml[a]		% Survival
		Untreated	Treated[b]	
GM30	*dcm*$^+$ *recA*$^+$	1.1×10^8	$(5.9 \pm 0.6) \times 10^7$	53.1 ± 5.8
BH127	*dcm*$^+$ *recA56*	1.4×10^6	$(6.7 \pm 3.5) \times 10^3$	0.48 ± 0.25
GM31	*dcm-6 recA*$^+$	9.4×10^6	$(5.3 \pm 1.5) \times 10^7$	56.0 ± 15.9
BH128	*dcm-6 recA56*	3.8×10^7	$(5.1 \pm 1.6) \times 10^6$	13.5 ± 4.1
RP4182	Δ*dcm recA*$^+$	1.2×10^8	$(5.7 \pm 1.6) \times 10^7$	48.0 ± 13.4
BH112	Δ*dcm recA56*	6.8×10^6	$(1.2 \pm 0.02) \times 10^5$	1.8 ± 0.03

[a] On LB plates.
[b] 5-azaC was used at 20 μg/ml.

(Table 3). If the potentially lethal form of the drug were 5-azauracil incorporated in the DNA in place of thymine, no correlation between the methylase overproduction and lethality would be expected. Furthermore, in this case, exogeneously added thymidine would be expected to suppress the lethal effect. This was found not to be the case (Table 6).

On the other hand, the ability of uracil to reverse the bacteriostatic effects of 5-azaC suggests that these effects are mediated through the conversion of the drug to 5-aza UMP. The product of the *upp* gene should convert uracil to UMP, and the UMP should competitively inhibit further phosphorylation of 5-aza UMP. This hypothesis is supported by the observation that in a *upp* mutant, uracil was unable to reverse the bacteriostatic effects (Fig. 4). The 5-aza UMP created in the cell after 5-azaC treatment is likely to result in the incorporation of 5-azauracil into RNA. Although it is not clear why the incorporation of 5-azauracil rather than 5-azacytosine into RNA led to the growth inhibition, it is known that treatment of *E. coli* with 5-azaC leads to the incorporation of 5-azauracil into RNA (8) and that the inhibition of protein synthesis caused by 5-azaC can be mimicked by the use of 5-azauridine (7).

In most studies concerning 5-azaC, the bacteriostatic effects of the drug have been considered undesirable but also unavoidable. Clearly, if one wishes to study the interaction between 5-azacytosine in DNA and DNA cytosine methylases in vivo and the subsequent response of the cell to the resulting DNA damage, it would be desirable to have the cell actively synthesizing proteins during this time. We have shown here that the addition of uracil to the growth medium may make this possible. By adding uracil to the growth medium, one should be able to perform experiments regarding the in vivo effects of the drug that were not possible before. Using this strategy, we have already isolated 5-azaC-resistant mutants of *E. coli* and have begun a quantitative study of possible SOS induction in the presence of 5-azaC.

Protein-DNA cross-links are caused by many DNA-damaging agents, including UV light and chemical carcinogens (1, 11, 37). Such complexes, if unrepaired, are likely to interfere with such essential cellular processes as replication, recombination, and transcription. Removal of methylase–5-azaC–DNA complexes by *E. coli* may be a useful model for the study of such repair processes.

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