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# Uracil-DNA Glycosylase Inhibitor of Bacteriophage PBS2: Cloning and Effects of Expression of the Inhibitor Gene in *Escherichia coli*

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**The uracil-DNA glycosylase inhibitor gene of bacteriophage PBS2 was cloned, and the effects of this inhibitor on *Escherichia coli* cells that contain uracil-DNA glycosylase activity were determined. A PBS2 genomic library was constructed by inserting *Eco*RI restriction fragments of PBS2 DNA into a plasmid pUC19 vector. The library was used to transform wild-type (*ung*$^+$) *E. coli*, and the presence of the functional inhibitor gene was determined by screening for colonies that supported growth of M13mp19 phage containing uracil-DNA. A clone was identified that carried a 4.1-kilobase *Eco*RI DNA insert in the vector plasmid. Extracts of cells transformed with this recombinant plasmid lacked detectable uracil-DNA glycosylase activity and contained a protein that inhibited the activity of purified *E. coli* uracil-DNA glycosylase in vitro. The uracil-DNA glycosylase inhibitor expressed in these *E. coli* was partially purified and characterized as a heat-stable protein with a native molecular weight of about 18,000. Hence, we conclude that the PBS2 uracil-DNA glycosylase inhibitor gene was cloned and that the gene product has properties similar to those from PBS2-infected *Bacillus subtilis* cells. Inhibitor gene expression in *E. coli* resulted in (i) a weak mutator phenotype, (ii) a growth rate similar to that of *E. coli* containing pUC19 alone, (iii) a sensitivity to the antifolate drug aminopterin similar to that of cells lacking the inhibitor gene, and (iv) an increased resistance to the lethal effects of 5-fluoro-2′-deoxyuridine. These physiological properties are consistent with the phenotypes of other *ung* mutants.**

Uracil residues may be introduced into DNA by two mechanisms; the incorporation of dUMP in place of dTMP during DNA synthesis and the deamination of cytosine residues in DNA. In *Escherichia coli*, incorporation of dUMP into DNA occurs efficiently in vitro by DNA polymerases I and III (29, 33) but is limited in vivo (33). This occurs in part because the level of intracellular dUTP pool is depleted by the *dut* gene product, deoxyuridine triphosphosphatase (dUTPase), which hydrolyzes dUTP to dUMP and $PP_i$ (13, 29). *E. coli* mutants that lack dUTPase activity (*dut*) are expected to exhibit an elevated concentration of dUTP which competes with dTTP for incorporation into DNA (13, 29, 36). Uracil residues that arise in DNA by incorporation or deamination reactions are normally removed by the *ung* gene product, uracil-DNA glycosylase, which cleaves the *N*-glycosylic bond between uracil and deoxyribose, thus producing an apyrimidinic site (20). It has been proposed that this enzyme initiates the first step in a base-excision repair pathway for duplex uracil-containing DNA (21, 31). *E. coli* mutants defective in both genes (*dut ung*) stably incorporate dUMP into DNA. When these double mutants are infected with bacteriophage M13, progeny phage are produced that contain single-stranded uracil-DNA. These progeny phage are subsequently restricted from growth in wild-type *E. coli* but are biologically active in an *E. coli ung* host (6, 16). This restriction phenomenon presumably involves the concerted action of uracil-DNA glycosylase plus apurinic-apyrimidinic endonucleases which catalyze strand breakage of single-stranded uracil-DNA, thus inactivating the phage genome. DNA fragmentation has been observed when uracil-DNA is introduced into procaryotic and eucaryotic cells containing these enzymes or when an imbalance in the intracellular dUTP-dTTP pool occurs (11, 14, 18, 34).

DNA of the *Bacillus subtilis* phage PBS2 is unique in that deoxyuridine replaces thymidine in the genome (30). Very early after infection of *B. subtilis*, several activities are induced that facilitate dUMP instead of dTMP incorporation into the phage DNA. Among these are dTMP 5′-phosphatase (27), dCTP deaminase (32), dUMP kinase (F. M. Kahar, Fed. Proc. 22:406, 1963), and dUTPase inhibitor (28), which function collectively to increase the size of the intracellular dUTP pool and decrease the dTTP pool. Consequently, dUMP rather than dTMP is incorporated into PBS2 DNA during phage replication, probably by a phage-induced DNA polymerase (12). In addition, a uracil-DNA glycosylase inhibitor is expressed which renders the host uracil-DNA glycosylase inactive (8). This inactivation results in permanent inclusion of uracil (36% total base composition) in PBS2 DNA. The uracil-DNA glycosylase inhibitor has been purified from PBS2-infected *B. subtilis* and characterized as an acidic protein of about 18,500 molecular weight (4). This heat-stable inhibitor has been reported to be active in vitro against uracil-DNA glycosylase isolated from *B. subtilis*, *E. coli*, *Micrococcus luteus*, *Saccharomyces cerevisiae*, and human KB cells (15).

Based on the above observations, we designed a strategy for cloning and expressing the PBS2 uracil-DNA glycosylase inhibitor gene in an *E. coli* strain that normally expressed the *ung* gene. In this report, we (i) describe the method for cloning the uracil-DNA glycosylase inhibitor gene from PBS2 DNA, (ii) demonstrate that expression of the cloned inhibitor gene transforms *E. coli* cells from an *ung*$^+$ to an *ung* mutant phenotype, (iii) show that the cloned inhibitor exhibits similar properties to that characterized from PBS2-infected *B. subtilis*, (iv) confirm that *E. coli* cells lacking uracil-DNA glycosylase activity exhibit a weak mutator phenotype, and (v) determine the effect of aminopterin and 5-fluoro-2′-deoxyuridine which cause a dUTP-dTTP pool imbalance on the survival of *E. coli* that express the uracil-DNA glycosylase inhibitor.

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(A preliminary report of this work appeared previously [Wang and Mosbaugh, Fed. Proc. 46:2258, 1987].)

## MATERIALS AND METHODS

**Strains and plasmids.** *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1*/pCJ105 ($Cm^r$)] and KT8052 [Δ(*lac pro*) *thi ara trpE9777 ung*-1/F' (*proAB, lacI*$^q$ZΔM15)] were provided by T. A. Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, N.C.). *E. coli* JM101 [Δ(*lac proAB*) *supE thi* ($r_k^+$ $m_k^+$/F' *traD36 proAB lacI*$^q$ZΔM15], JM107 [Δ(*lac pro* AB) *thi gyrA96 endA1 hsdR17 relA1 supE44* ($r_k^-$ $m_k^+$) $\lambda^-$/F' *traD36 proAB lacI*$^q$ZΔM15], bacteriophage M13mp19, and the plasmid vector pUC19 were obtained from Bethesda Research Laboratories. Bacteriophage PBS2 and its host *B. subtilis* 168 (*trp*) were from S. M. Linn (University of California, Berkeley).

**Reagents and materials.** Ampicillin, chloramphenicol, nalidixic acid, 5-fluoro-2'-deoxyuridine, and aminopterin were purchased from Sigma Chemical Co. Isopropylthio-β-galactoside (IPTG) and 5-dibromo-4-chloro-3-indolylgalactoside (X-gal) came from Bethesda Research Laboratories, and repliplate colony transfer pads from FMC Corp. Restriction endonuclease *Eco*RI and T4 DNA ligase were from New England Biolabs, and [$^3$H]dUTP was from Amersham Corp. *E. coli* uracil-DNA glycosylase (fraction V) was purified by the method of Lindahl et al. (20). Calf thymus DNA containing [$^3$H]uracil residues was prepared by the method of Domena and Mosbaugh (5).

**Media.** The M9 minimal medium described by Miller (25) was supplemented with 0.001% thiamine and 0.01% ampicillin. YT medium was prepared as described by Kunkel et al. (17) and, when indicated, was supplemented with 0.25 μg of uridine per ml. YT plates were prepared similarly, except 1.5% Bacto-Agar (Difco Laboratories) was included. TYN-ampicillin plates contained 1% tryptone, 1% yeast extract, 0.5% NaCl, 0.01% ampicillin, and 1.5% Bacto-Agar. TYG medium containing 2.5% tryptone, 2% yeast extract, 3% glucose, and 0.3% $Na_2HPO_4$ was supplemented with tryptophan to a final concentration of 0.5 mM.

**Preparation of M13mp19 phage containing uracil-DNA.** Bacteriophage M13mp19 containing uracil-DNA was prepared essentially as described by Kunkel et al. (17); some thymine residues in the phage DNA were replaced by uracil. *E. coli* CJ236 cells were grown at 37°C with vigorous shaking in YT medium supplemented with 0.25 μg of uridine per ml. After growing to a density of $4 \times 10^8$ cells per ml, M13mp19 phage was added at a multiplicity of infection of 5. At 7 h postinfection, the culture was centrifuged at 5,000 × *g* for 15 min. The progeny phage in the supernatant fraction was titered on both *E. coli* JM101 (*ung*$^+$) and KT8052 (*ung*) strains to determine the host range of viability. Phage containing uracil-DNA were shown to have almost normal biological activity in the *ung*$^-$ host but were restricted from growth in the *ung*$^+$ host. Generally, these phage titers were about $10^5$-fold lower on JM101 than on KT8052. These M13mp19 phage shown to be restricted by the *ung*$^+$ host were used as a biological probe to screen the PBS2 genomic library in *E. coli* JM101 for clones expressing the PBS2 uracil-DNA glycosylase inhibitor gene.

**Construction of the PBS2 genomic library.** Motile log-phase *B. subtilis* 168 cells were grown at 37°C in TYG medium (200 ml) and infected with PBS2 at a multiplicity of infection of 5. The culture was incubated for 3 h at 37°C with vigorous shaking to allow lysis. Complete lysis was achieved by the addition of chloroform (2 ml) and by shaking at 37°C for 20 min. Subsequent procedures were performed at 0 to 4°C unless otherwise indicated. The lysate was centrifuged at 8,000 × *g* for 15 min to remove debris. PBS2 phage DNA was then isolated from the supernatant fraction by a modification of the method of Gates and Linn (9). PBS2 phage were centrifuged at 60,000 × *g* for 4 h in a Beckman 21 rotor. The phage pellet was suspended in 3 ml of SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) solution and centrifuged again at 15,000 × *g* for 15 min. The supernatant fraction was loaded onto preformed gradients made by layering equal volumes of CsCl at densities of 1.7, 1.5, and 1.3 g/cm$^3$ in centrifuge tubes. Centrifugation was at 175,000 × *g* for 90 min in a Beckman SW50.1 rotor at 20°C. The phage band was collected and applied to a Bio-Gel A-15m column (4.9 cm$^2$ by 45 cm; Bio-Rad Laboratories), which was equilibrated and eluted with SSC solution. PBS2 phage were detected in the void volume by absorption at 260 nm and titered on *B. subtilis* 168 as described by Price and Cook (26). Pooled phage fractions were then centrifuged at 368,000 × *g* for 75 min in a Beckman type 60 rotor. The phage pellet was suspended in SSC solution, adjusted to 0.1% sodium dodecyl sulfate, and extracted with 0.2 M Tris hydrochloride (pH 7.5)-saturated phenol. After the aqueous phase was adjusted to 0.5 M NaCl, it was extensively dialyzed against 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–1 M NaCl, then against 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA, and finally against 10 mM Tris hydrochloride (pH 7.5). PBS2 DNA (6 μg) was digested with 30 U of *Eco*RI endonuclease in a reaction mixture (50 μl) that contained 100 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, and 100 μg of bovine serum albumin per ml. Incubation was for 4 h at 37°C. The reaction was stopped by heating at 65°C for 15 min, and the DNA was precipitated in ethanol and suspended in buffer [10 mM Tris hydrochloride (pH 8.0), 1 mM EDTA]. Ligation of the PBS2 DNA fragments into the *Eco*RI site of pUC19 DNA was performed in a reaction mixture (22 μl) containing 50 mM Tris hydrochloride (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, 50 μg of bovine serum albumin per ml, 400 ng of PBS2 DNA fragments, 80 ng of linear pUC19 DNA, and 40 U of T4 DNA ligase. Incubation was for 14 h at 16°C. Competent *E. coli* KT8052 cells were transformed with this DNA ligation mixture, and transformants were selected on TYN-ampicillin plates as described by Maniatis et al. (24). Recombinant plasmid DNA was isolated from individual colorless colonies by the alkaline lysis procedure originally described by Birnboim and Doly (1). These plasmids were designated pZW and then individually transformed into competent *E. coli* JM101 cells by the transformation procedure indicated above. Colorless colonies that grew on TYN-ampicillin plates were picked and transfered to master plates that contained the same medium.

**Preparation of cell extracts.** *E. coli* cells were grown in 1,000 ml of TYN medium (supplemented with ampicillin when appropriate) to the late-log phase and harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. Cells were washed and pelleted by centrifugation, and the cell pellet was suspended in 100 ml of 10 mM Tris hydrochloride (pH 8.0). These cells were then disrupted by sonication using 10 to 12 pulses of 30 s each (120 W, large probe) with a cell disruption sonifier model 350 (Branson Sonic Power Co.). The sonicated solution was centrifuged at 7,700 × *g* for 15 min at 4°C, and pellet was suspended in 50 ml of 10 mM Tris hydrochloride (pH 8.0) and sonicated again as before. The sonicated suspensions were combined and then centrifuged

at 17,000 × *g* for 15 min at 4°C, and the resulting supernatant was used as the cell extract.

**Purification of uracil-DNA glycosylase inhibitor.** All procedures were performed at 0 to 4°C unless otherwise indicated. A cell extract of *E. coli* JM101(pZW177) was prepared as described above and designated fraction 1.

**(i) Ammonium sulfate precipitation.** Fraction 1 was brought to 45% saturation with ammonium sulfate by slowly adding salt to the solution over a 30-min period while stirring on ice. After an additional 30 min of stirring, the precipitate was removed by centrifugation at 15,000 × *g* for 15 min. The supernatant fraction was then brought to 75% saturation with ammonium sulfate, and the proteins precipitating at this concentration were collected by centrifugation as before. The pellet was suspended in 20 ml of TED buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol). After extensive dialysis in the same buffer the sample was designated fraction 2.

**(ii) Thermal precipitation.** Fraction 2 was heated at 100°C for 15 min to denature exogenous proteins. The precipitate that formed during this treatment was then removed by centrifugation at 17,000 × *g* for 15 min, and the supernatant solution constituted fraction 3.

**(iii) DEAE-cellulose chromatography.** A DEAE-cellulose (Whatman DE-52) column (2.3 $cm^2$ by 23 cm) was equilibrated with TED buffer. Fraction 3 was loaded, and the column was washed with 160 ml of equilibration buffer at a flow rate of approximately 40 ml/h. The uracil-DNA glycosylase inhibitor was eluted with a 200-ml linear gradient from 0 to 1 M NaCl in TED buffer. Fractions (4.2 ml) were assayed for uracil-DNA glycosylase inhibitor activity by using 25 μl samples; the peak activity eluted at 325 mM NaCl. Active fractions were pooled and represented fraction 4.

**(iv) Sephadex G-75 chromatography.** A sample (3 ml) of fraction 4 was applied to a Sephadex G-75 column (1.8 $cm^2$ by 58 cm) equilibrated in 50 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–1 mM dithiothreitol–100 mM NaCl–10% (w/v) glycerol. The inhibitor protein was eluted with a constant flow rate (13 ml/h) with the equilibration buffer. Peak fractions were pooled and designated fraction 5.

**Uracil-DNA glycosylase inhibitor assay.** Standard reaction mixtures (100 μl) contained 70 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–KOH (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 9.2 nmol of calf thymus DNA containing [$^3$H]uracil (266 cpm/pmol of uracil), 0.04 to 0.1 U of *E. coli* uracil-DNA glycosylase, and various amounts of uracil-DNA glycosylase inhibitor as indicated in the figure legends. After incubation for 30 min at 37°C, the reactions were terminated on ice with 250 μl of 10 mM ammonium formate (pH 4.2). A sample (300 μl) was then applied to a Bio-Rad AG 1-X8 (formate form) column equilibrated with 10 mM ammonium formate (pH 4.2), and the [$^3$H]uracil which did not bind to the resin was eluted with 1.7 ml of the same buffer. Four 500-μl fractions were collected, and the radioactivity was measured after the addition of 5 ml of Ready-Solv HP/b (Beckman). The endogenous uracil-DNA glycosylase activity was determined in the absence of added uracil-DNA glycosylase inhibitor. One unit of uracil-DNA glycosylase inhibitor inactivates one unit of uracil-DNA glycosylase in the above reaction. Exogenous uracil-DNA glycosylase activity was measured as described above, except that the endogenous glycosylase was omitted from the reaction mixture. One unit of uracil-DNA glycosylase is defined as the amount that releases 1 nmol of uracil per h under standard reaction conditions.

**Determination of spontaneous mutation frequencies.** Cultures of *E. coli* JM101(pUC19) or JM101(pZW177) were started from a single colony in 2 ml of YT medium supplemented with 0.01% ampicillin. After growing overnight at 37°C to reach the stationary phase, cells were diluted and plated on YT-ampicillin plates to determine the number of total viable cells. One sample (250 μl) of the overnight culture was plated on YT-ampicillin plates containing 20 μg of nalidixic acid per ml, and a second sample (100 μl) was plated on YT-ampicillin plates containing 5 μg of chloramphenicol per ml. Nalidixic acid-resistant cells were counted after overnight incubation at 37°C, whereas chloramphenicol-resistant cells were scored after incubation for 24 to 36 h.

**Determination of aminopterin and 5-fluoro-2'-deoxyuridine sensitivity.** *E. coli* JM101 cells containing pUC19 or pZW177 were grown to the stationary phase at 37°C in M9 minimal medium supplemented with 0.001% thiamine and 0.01% ampicillin. After cells were diluted 100-fold in the above medium, which also contained 10 μM hypoxanthine, 20 μg of glycine per ml, and 20 μg of serine per ml, the cells were grown for 3 h to ensure that the cells were in the logarithmic phase of growth. Then various amounts of aminopterin or 5-fluoro-2'-deoxyuridine were added to the cultures as indicated in the figure legends. After growth at 37°C for another 4 h, cells were appropriately diluted and immediately plated on M9 minimal medium plates supplemented with 0.001% thiamine and 0.01% ampicillin. The number of viable cells was measured after 48 h of incubation at 37°C, and the percent survival was determined relative to the number of viable cells observed in cultures that were not treated with the toxic compounds.

## RESULTS

**Cloning of PBS2 uracil-DNA glycosylase inhibitor gene.** An outline of the approach used to clone the PBS2 uracil-DNA glycosylase inhibitor gene from PBS2 DNA is shown in Fig. 1. A PBS2 genomic library was constructed by inserting *Eco*RI restriction fragments of uracil-DNA into the *Eco*RI restriction site of pUC19 DNA. Complete digestion of PBS2 DNA with *Eco*RI endonuclease generated about 43 resolvable DNA fragments ranging from $1.3 \times 10^5$ to $1.5 \times 10^7$ molecular weight as determined by 0.7% agarose gel electrophoresis. Insertion of these DNA fragments into the pUC19 vector disrupted the *lacZ* α-coding sequence for the β-galactosidase α-peptide. This allowed us to screen for recombinant plasmids based on their inability to produce functional β-galactosidase by α-complementation once plasmid DNA was transformed into *E. coli* KT8052 cells. The recombinant plasmids transformed this strain, a uracil-DNA glycosylase mutant, with a significantly higher efficiency (8,800 transformants per μg of vector DNA) than they transformed *E. coli* (250 transformants per μg of vector DNA) that expressed this enzyme. We assume that the reduced ability to transform the wild-type strain was caused by degradation of uracil-DNA contained in the PBS2 DNA insert. Although the exact mechanism for this decrease in transformation efficiency remains unknown, it did not appear to involve the restriction-modification system of *E. coli* K, since these recombinant plasmids did not efficiently transform *E. coli* JM107 ($r_k^-$ $m_k^+$) which lacked the restriction system. Transformants of *E. coli* KT8052 were identified as colorless colonies on TYN plates containing ampicillin, IPTG, and X-gal. Plasmid DNA was isolated from 350 independent colonies which were ampicillin resistant and lacked β-galactosidase activity (α-complementation negative). This set of recombinant plasmids were referred to as

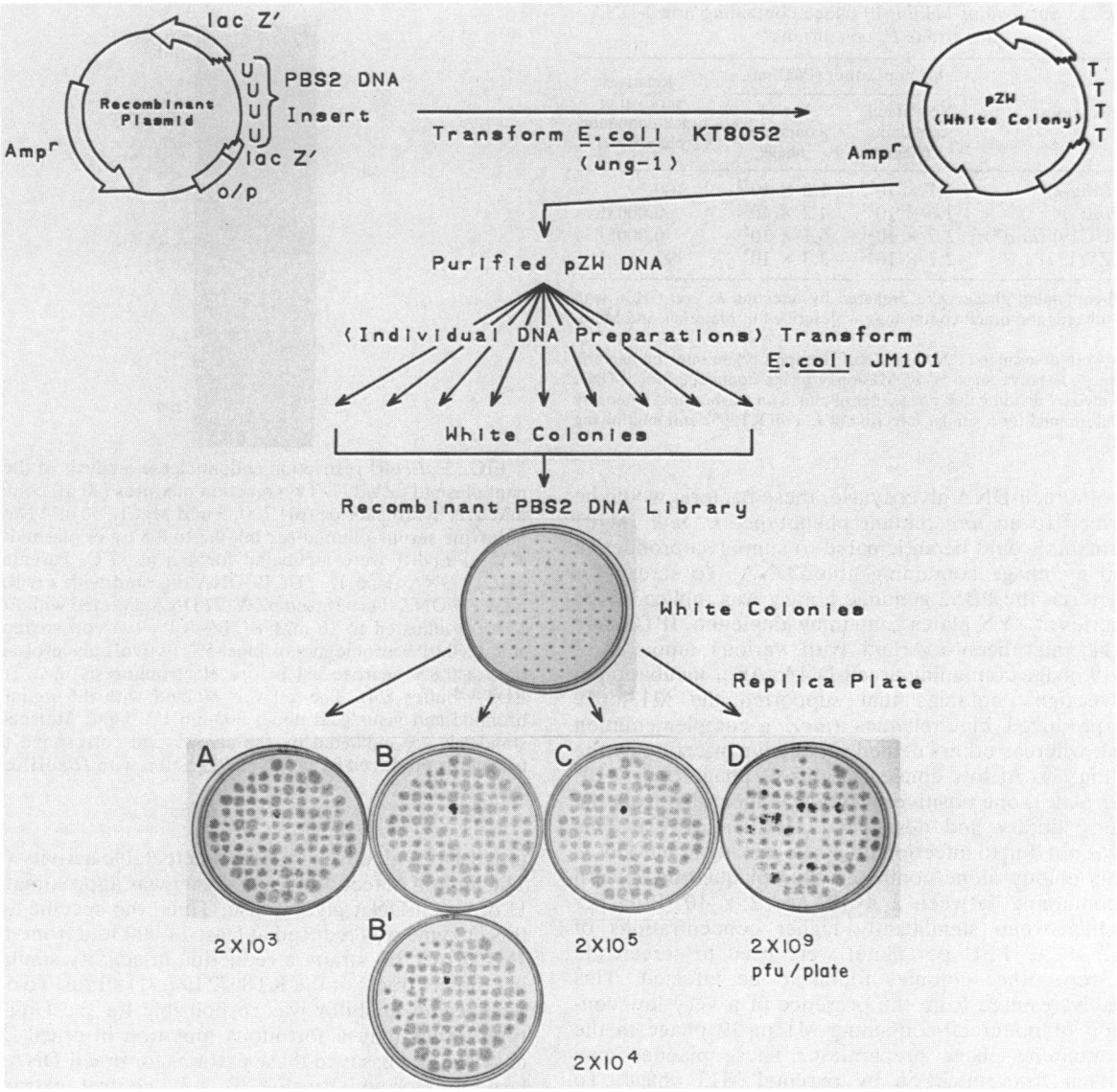


FIG. 1. Cloning strategy for isolating the PBS2 uracil-DNA glycosylase inhibitor gene.

pZW, and individual plasmids were transformed into *E. coli* JM101 cells that expressed uracil-DNA glycosylase. Ampicillin-resistant transformants were selected as before, and one representative colorless colony was isolated from each transformation. This bacteria containing pZW were maintained on TYN plates containing ampicillin and represented the PBS2 genomic library.

An in vivo screening procedure was developed to identify colonies that expressed the PBS2 uracil-DNA glycosylase inhibitor. This unique technique was derived from the previous observation that M13 phage grown in an *E. coli* (*dut ung*) strain produced progeny phage that inefficiently propagate in wild-type *E. coli* but productively infect *E coli* (*ung*) strains (6, 16). During a productive infection, β-galactosidase α-peptide, which is coded by the M13mp19 genome, is expressed and complements the defective β-galactosidase gene product of the host. Hence, a productive infection will result in a blue plaque when cells are grown on plates containing IPTG and X-gal. In contrast, plaque formation rarely occurs when wild-type *E. coli* cells containing uracil-DNA glycosylase are infected. To perform the screening technique M13mp19 phage were first grown in *E. coli* CJ236 (*dut ung*) to produce phage DNA in which a portion of the thymine residues were replaced by uracil (16). These phage along with parental (nonuracil-containing) phage were titered on both *E. coli* KT8052 and JM101 strains (Table 1). As indicated by a $2.9 \times 10^5$-fold lower efficiency of survival, the M13mp19 phage containing uracil-DNA was restricted from growth on the host that expressed uracil-DNA glycosylase. On the other hand, M13mp19 phage lacking uracil-DNA was found to infect each strain with approximately equal efficiency. Hence this difference was not due to phage resistance of the wild-type strain. Similarly, *E. coli* JM101 with or without plasmid pUC19 was found to only restrict growth of uracil-containing phage. Based on these observations, we predicted that M13mp19 phage containing uracil-DNA would not generally propagate in *E. coli* JM101 carrying pZW unless these cells contained a plasmid that expressed functional PBS2 uracil-DNA glycosylase inhibitor. We hypothesized that if this gene were expressed and inactivated

TABLE 1. Survival of M13mp19 phage containing uracil-DNA in various *E. coli* strains[a]

| Host strain | M13mp19 titer (PFU/ml) | | Relative survival of uracil-containing phage[b] (%) |
|---|---|---|---|
| | Non-uracil-containing phage | Uracil-containing phage | |
| KT8052 (*ung*-1) | $1.1 \times 10^{12}$ | $1.2 \times 10^{11}$ | 100 |
| JM101 (*ung*$^+$) | $1.6 \times 10^{12}$ | $4.2 \times 10^{5}$ | 0.00035 |
| JM101(pUC19) (*ung*$^+$) | $2.7 \times 10^{12}$ | $6.1 \times 10^{5}$ | 0.00051 |
| JM101(pZW177) | $2.7 \times 10^{12}$ | $2.3 \times 10^{11}$ | 192 |

[a] Uracil-containing phage were prepared by infecting *E. coli* CJ236 with M13mp19 phage (non-uracil containing) as described in Materials and Methods.

[b] Titers were determined after growth of the appropriate infected bacteria on YT plates. Relative survival of M13mp19 phage containing uracil-DNA was calculated by dividing that phage titer obtained on various host strains by the titer determined for a similar infection of *E. coli* KT8052 and multiplying by 100.

the *E. coli* uracil-DNA glycosylase, these bacteria would be transformed to an *ung* mutant phenotype. *E. coli* JM101 transformants would be anticipated to support a productive infection by phage containing uracil-DNA. To screen for such bacteria, the PBS2 genomic library was replica plated onto a series of TYN plates containing ampicillin, IPTG, and X-gal that had been overlaid with various amounts of M13mp19 phage containing uracil-DNA. After incubation at 37°C overnight, colonies that supported the M13mp19 growth produced blue plaques (*lacZ* α-complementation positive), whereas others denied productive infection by the phage (Fig. 1). At low concentrations of phage ($<2 \times 10^5$ PFU per plate), one positive colony was identified from the 350-colony library and designated JM101(pZW177). This result was not due to infection by phage lacking uracil-DNA, since this colony alone contained blue plaques on several plates containing between $2 \times 10^3$ and $2 \times 10^5$ PFU per plate. Only when significantly higher concentrations of phage ($2 \times 10^9$ PFU per plate) were used to screen the library were other colonies found to be infected. This presumably resulted from the presence of a very low concentration of nonuracil-containing M13mp19 phage in the uracil-containing phage preparation. These plaques may have arisen from infection by parental M13 phage. To establish this point more definitively and to determine what percent, if any, the uracil-containing phage were being restricted from growth on *E. coli* JM101(pZW177), we titered phage on this host (Table 1). We found that these cells efficiently supported the growth of M13mp19 phage containing uracil-DNA, whereas the titer of parental phage remained unchanged when compared with the results obtained for *E. coli* JM101 containing pUC19. This result would be expected if the uracil-DNA glycosylase inhibitor gene product efficiently inactivated the host uracil-DNA glycosylase in vivo.

**EcoRI restriction endonuclease analysis of the recombinant plasmid pZW177.** The cells which were transformed by pZW177 and supported the productive infection of M13mp19 phage containing uracil-DNA were colony purified and used to isolate pZW177 DNA. The recombinant plasmid was isolated and digested with *Eco*RI restriction endonuclease, which produced two linear DNA fragments (Fig. 2). One fragment corresponded to that of linear pUC19 DNA, and the other corresponded to a 4.1-kilobase fragment of PBS2 DNA.

**Enzyme activity of *E. coli* JM101(pZW177) extracts.** Cell extracts of the JM101(pZW177) strain assayed in vitro for

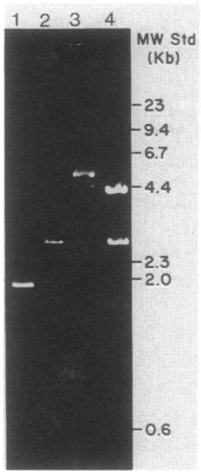


FIG. 2. *Eco*RI restriction endonuclease analysis of the recombinant plasmid pZW177. Two reaction mixtures (20 μl) containing 100 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 100 μg of bovine serum albumin per ml, 0.2 to 0.8 μg of plasmid DNA and 4 U of *Eco*RI were incubated for 4 h at 37°C. Parental plasmid pUC19 DNA (lane 1), pUC19 DNA digested with *Eco*RI (lane 2), pZW177 DNA (lane 3), and pZW177 DNA digested with *Eco*RI (lane 4) were adjusted to 10 mM EDTA–0.1% (wt/vol) sodium dodecyl sulfate–0.01% bromophenol blue–5% (wt/vol) glycerol and loaded onto a 0.8% agarose gel before electrophoresis in a Tris-borate-EDTA buffer (24). The gel was stained with 0.5 μg/ml ethidium bromide and visualized under 300-nm UV light. Molecular weight standards are indicated by the arrows and correspond to λ DNA fragments produced by complete digestion with *Hin*dIII endonuclease.

uracil-DNA glycosylase lacked detectable activity (Table 2). The limit of detection for this assay was approximately 0.001 U of uracil-DNA glycosylase. Thus, the specific activity of this enzyme was reduced at least 14,000-fold from that of the JM101(pUC19) strain, a reduction in activity similar to that in crude extracts of the KT8052 (*ung-1*) strain. To determine whether an inhibitor was responsible for the Ung$^-$ phenotype rather than a fortuitous mutation in uracil-DNA glycosylase, we assayed these extracts for uracil-DNA glycosylase inhibitor activity (Fig. 3). As a control, extracts of the *ung-1* mutant were added to reactions containing purified *E. coli* uracil-DNA glycosylase. As expected, the extract from the *ung* mutant did not contain any inhibitor activity, since this phenotype results from a mutation in the uracil-DNA glycosylase gene. However, extracts of *E. coli* JM101(pZW177) were shown to inhibit the exogenous *E. coli* uracil-DNA glycosylase activity in a linear, concentration-dependent

TABLE 2. Uracil-DNA glycosylase activity in various *E. coli* cell extracts[a]

| Strain | Uracil-DNA glycosylase activity (U/ml) | Protein (mg/ml) | Sp act (U/mg) |
|---|---|---|---|
| KT8052 (*ung*-1) | 0.045 | 6.9 | 0.0065 |
| JM101 (*ung*$^+$) | 744 | 8.1 | 92 |
| JM101(pUC19) (*ung*$^+$) | 720 | 8.0 | 90 |
| JM101(pZW177) | 0.052 | 8.1 | 0.0064 |

[a] *E. coli* were grown to the late-log phase in 500 ml of TYN medium, and cell extracts were prepared as described in Materials and Methods. After centrifugation at 17,000 × *g* for 15 min, the supernatant fraction was assayed for uracil-DNA glycosylase activity, and protein was determined by the method of Bradford (2) with bovine serum albumin as the standard.

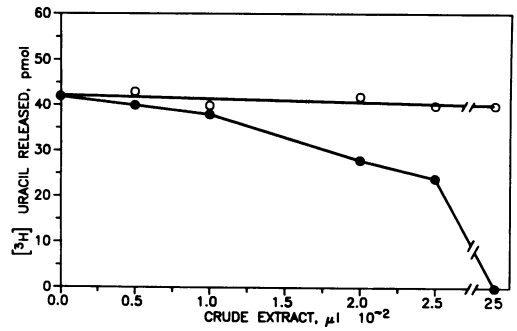


FIG. 3. Inhibition of purified *E. coli* uracil-DNA glycosylase by the cellular extract of *E. coli* JM101 containing plasmid pZW177. Cell extracts of *E. coli* KT8052 (○) and JM101(pZW177) (●) were prepared as described in Materials and Methods. Various amounts of each extract were added to a standard uracil-DNA glycosylase inhibitor reaction. Each assay containing 0.085 U of *E. coli* uracil-DNA glycosylase was incubated for 30 min at 37°C. The reactions were then terminated on ice, and 250 μl of 10 mM ammonium formate (pH 4.2) was added. A portion of each reaction mixture was then applied to a Bio-Rad AG 1-X8 column, and the [$^3$H]uracil which did not bind to the resin was measured with a scintillation spectrometer.

manner. We calculate that this extract contained about 239 U of uracil-DNA glycosylase inhibitor activity per mg of protein. The data indicate that unlike the *ung-1* mutant, the JM101 (pZW177) strain lacked uracil-DNA glycosylase activity because of the presence of a uracil-DNA glycosylase inhibitor. Furthermore, the specific activity of the inhibitor in *E. coli* JM101(pZW177) appears to be about 2.7-fold higher than the uracil-DNA glycosylase activity (90 U/mg) in extracts of *E. coli* JM101(pUC19). This explains the absence of uracil-DNA glycosylase activity in *E. coli* JM101(pZW 177). Thus we concluded that the PBS2 uracil-DNA glycosylase inhibitor gene was cloned and expressed in these cells.

**Purification and properties of the cloned uracil-DNA glycosylase inhibitor.** The product of the cloned inhibitor gene was partially purified to compare its properties with those described for the uracil-DNA glycosylase inhibitor obtained from PBS2 phage-infected *B. subtilis* (4). Our purification procedure involved (i) precipitation of the inhibitor from a *E. coli* JM101(pZW177) extract with 45 to 75% saturated ammonium sulfate, (ii) heat treatment at 100°C for 15 min, (iii) DEAE-cellulose chromatography, and (iv) molecular sieving on a Sephadex G-75 column. The most pure fraction had been purified more than 100-fold over the crude extract. Several lines of evidence indicate that the uracil-DNA glycosylase inhibitor derived from *E. coli* JM101(pZW177) cells exhibited properties similar to the inhibitor isolated from PBS2-infected *B. subtilis* (4). First, both inhibitors are nondialyzable and unusually heat stable. We recovered 110% of inhibitor activity after treatment at 100°C for as long as 30 min. Second, the cloned gene product was shown to be a protein which was sensitive to proteinase K treatment. Incubation with 200 μg of proteinase K per ml for 60 min at 60°C totally inactivated this inhibitor, a result similar to that described by Cone et al. (4). Third, both are acidic polypeptides that bind to DEAE-cellulose. We found that the cloned inhibitor eluted from the DEAE-cellulose column at about 325 mM NaCl (Fig. 4A). The inhibitor isolated by Friedberg and co-workers was reported to elute from a DEAE-Sephadex column at about 250 mM NaCl, under slightly different conditions (4). Fourth, the native molecular weight of uracil-DNA glycosylase inhibitor produced in *E. coli* was determined by gel filtration analysis on a Sephadex G-75 column to be approximately 18,000 (Fig. 4B). This value was in good agreement with the molecular weight of 18,500 ± 500 reported by Cone et al. (4) for the natural inhibitor. Based on these properties, the inhibitors produced in *E. coli* and *B. subtilis* appear to be very similar proteins.

**Effect of uracil-DNA glycosylase inhibitor on mutation rates.** Since *E. coli ung* mutants exhibit elevated spontaneous mutation rates, we tested whether *E. coli* JM101 that contained the uracil-DNA glycosylase inhibitor showed a similar phenotype. Mutation to resistance to nalidixic acid and chloramphenicol was measured in isogenic strains JM101(pUC19) and JM101(pZW177). The spontaneous mutation frequency was 12.8-fold and 9.2-fold higher for resistance to nalidixic acid and chloramphenicol, respectively, in the JM101(pZW177) strain (Table 3). These results indicate that the uracil-DNA glycosylase inhibitor promotes a weak mutator phenotype which is similar to that observed for the *E. coli ung* strain (6).

**Effect of aminopterin and 5-fluoro-2′-deoxyuridine on survival of *E. coli* JM101(pZW177).** The antifolate agent aminopterin is a competitive inhibitor of dihydrofolate reductase, which depletes the pool of folate cofactor necessary for dTMP synthesis from dUMP. Treatment of cells with agents that generally upset the balance of the dTTP pool, in favor of increasing the dUTP pool, causes thymineless death (14, 33, 38). The cytotoxicity of antifolate agents in mammalian cells has been positively correlated in vivo with significant dUMP incorporation into DNA and excessive strand breakage of cellular DNA (11, 14, 18). Thus, a hypothesis has been presented which suggests that cytotoxicity results from DNA fragmentation induced by uracil residues in DNA (10, 14). One mechanism for generating strand breaks in uracil-DNA might utilize uracil-DNA glycosylase to produce baseless sites that lead to strand scission. As the first step toward testing such a hypothesis in bacteria, we measured the susceptibility of *E. coli* isogenic strains JM101(pUC19) and JM101(pZW177) to aminopterin. One would predict that the JM101(pZW177) strain, in which uracil-DNA glycosylase-initiated DNA strand breakage should be blocked, would be more resistant to aminopterin than the JM101(pUC19) strain. Contrary to this prediction, JM101(pZW177) was almost equally susceptible to aminopterin as the strain containing uracil-DNA glycosylase activity (Fig. 5A).

Since the effect of aminopterin on cellular metabolism is pleiotropic, we also examined the effect on survival of 5-fluoro-2′-deoxyuridine, a more specific inhibitor of thymidylate synthetase. It has been shown that 5-fluoro-2′-deoxyuridine treatment also resulted in an imbalance of cellular dUTP-dTTP pools and DNA fragmentation (38). In this regard, we examined the lethal effects of 5-fluoro-2′-deoxyuridine on the *E. coli* isogenic strains JM101(pUC19) and JM101(pZW177) to investigate possible roles of uracil-DNA glycosylase in the process of cell death induced by depletion of dTTP pool. Unlike the case with aminopterin treatment, JM101(pZW177) bacteria were slightly more resistant to 5-fluoro-2′-deoxyuridine than cells that lacked the inhibitor gene (Fig. 5B). Both strains had almost identical generation times of about 70 min at 37°C in M9 minimal medium. Thus, the difference in survival was probably not due to differences in the extent of DNA replication during the treatment period.

## DISCUSSION

The results demonstrate that we have cloned the uracil-DNA glycosylase inhibitor gene from bacteriophage PBS2

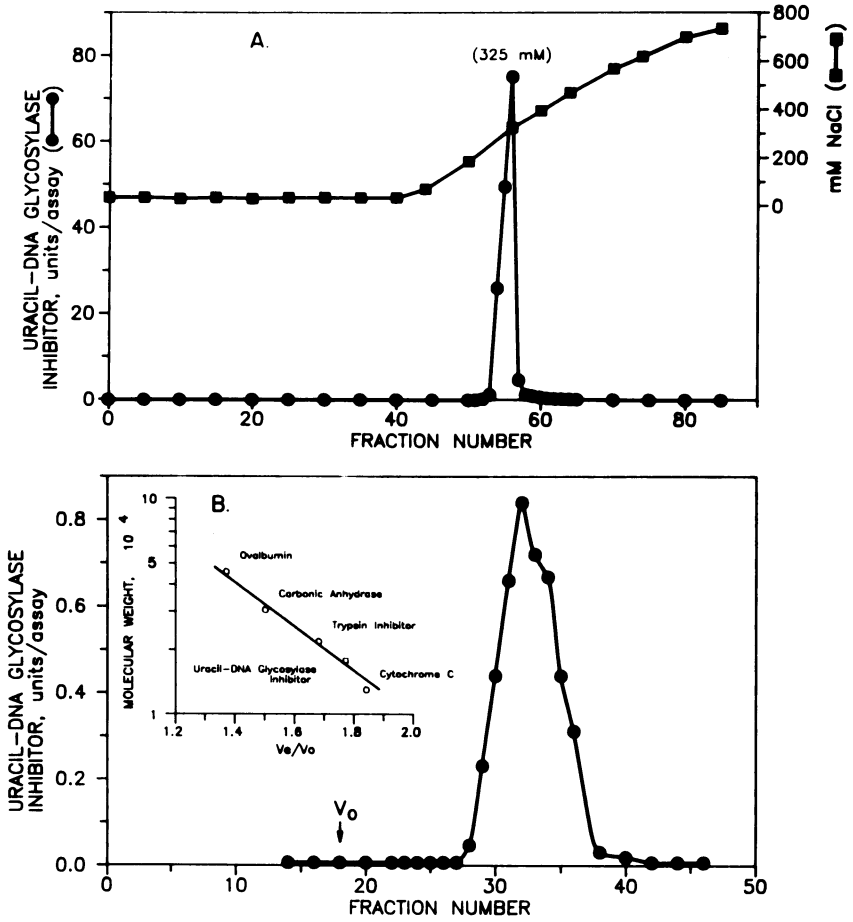


FIG. 4. Purification and molecular weight determination of cloned uracil-DNA glycosylase inhibitor gene product. (A) Fraction 3 uracil-DNA glycosylase inhibitor (34,000 U) was loaded onto and then eluted from a DEAE-cellulose column as described in Materials and Methods. Fractions were assayed for inhibitor activity as shown, and peak fractions were pooled to represent fraction 4. (B) Fraction 4 uracil-DNA glycosylase inhibitor (150 U) was applied to a Sephadex G-75 column, eluted, and assayed for inhibitor activity as described in Materials and Methods. The arrow indicates the location of the void volume ($V_0$) and was determined by using blue dextran 2000. The column was calibrated with ovalbumin ($M_r$ 45,000), carbonic anhydrase ($M_r$ 30,000), trypsin inhibitor ($M_r$ 21,000), and cytochrome *c* ($M_r$ 12,500) as molecular weight standards. A plot of molecular weight versus $V_e/V_0$ ratio for the protein standards (○) is shown in the insert along with the cloned uracil-DNA glycosylase inhibitor (□).

and expressed its gene product in *E. coli*. This was suggested by (i) the ability of M13mp19 phage containing uracil-DNA to productively infect JM101(pZW177) cells, (ii) the absence of uracil-DNA glycosylase activity in extracts of these cells, (iii) the presence of an inhibitor protein in these cells that inactivates purified *E. coli* uracil-DNA glycosylase in vitro, and (iv) the phenotypic similarities between this clone and previously described *ung* mutants. It was perhaps not surprising that a positive clone was identified that exhibited significant reduction in uracil-DNA glycosylase activity (0.006% residual activity), since phage survival is apparently dependent on a relatively nonleaky phenotype. Duncan et al. (6) initially made this observation by using T4 phage containing numerous uracil residues in place of thymine in phage DNA to infect *E. coli*. He found that these phage were viable only in *ung* mutants. In addition, only the most enzyme-deficient alleles *ung-1* (0.02% residual activity) and *ung-2* (0.7% residual activity) supported phage infection. We attribute the abortive phage infections to uracil-DNA glycosylase-initiated fragmentation of uracil-DNA.

We have taken advantage of this strong biological selection against survival of M13mp19 phage containing uracil-DNA to develop a biological probe for screening a PBS2 genomic library in *E. coli* for clones that express the uracil-DNA glycosylase inhibitor of bacteriophage PBS2. We successfully reasoned that if the inhibitor gene were expressed in *E. coli* these cells would be transformed to a uracil-DNA glycosylase deficient phenotype. Our results concerning the relative survival of M13mp19 phage containing uracil-DNA in the clone expressing the inhibitor gene versus the isogenic

TABLE 3. *E. coli* JM101 (*ung*$^+$) containing plasmid pZW177 exhibit a mutator phenotype[a]

| Drug resistance mutation | Mutants per $10^9$ viable cells | | Ratio[b] |
|---|---|---|---|
| | JM101(pUC19) | JM101(pZW177) | |
| Nalidixic acid | 3.2 ± 1.2 | 41 ± 19 | 12.8 |
| Chloramphenicol | 120 ± 50 | 1,100 ± 400 | 9.2 |

[a] Spontaneous mutation frequencies were determined as described in the Materials and Methods. Frequencies were calculated as the number of resistant colonies formed per $10^9$ viable cell and was determined after 13 and 9 separate experiments with nalidixic acid and chloramphenicol, respectively.

[b] Ratio of results with strain JM101(pZW177) to those with strain JM101(pUC19).

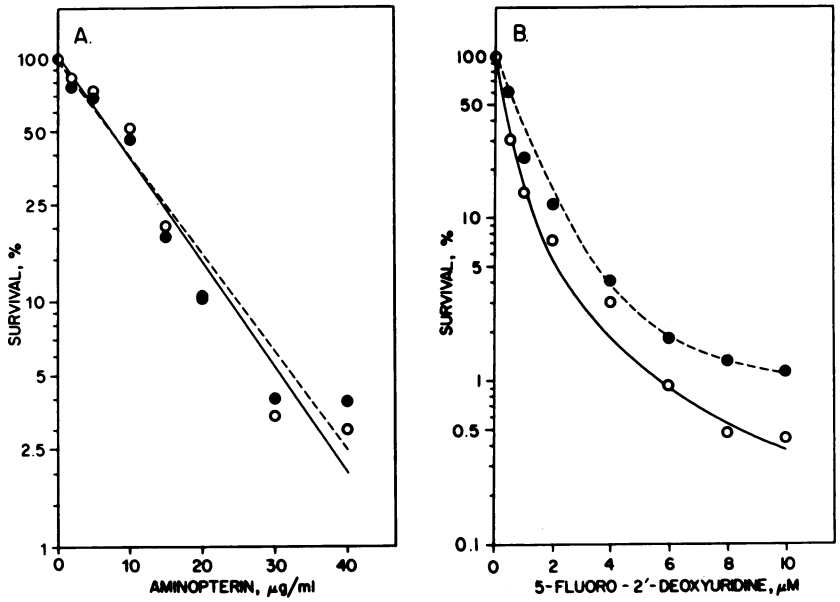


FIG. 5. Effect of pZW177 on survival of *E. coli* after treatment with aminopterin or 5-fluoro-2'-deoxyuridine. *E. coli* JM101 cells containing pUC19 (○) or pZW177 (●) were treated for 4 h during the logarithmic phase of growth with various amounts of aminopterin (A) or 5-fluoro-2'-deoxyuridine (B) as described in Materials and Methods. Then cells were appropriately diluted and immediately spread on M9 minimal plates supplemented with 0.001% thiamine and 0.01% ampicillin. Viable cells were counted, and the percent survival was determined relative to the number of viable cells in untreated cultures. The survival curves of JM101(pUC19) (—) and JM101(pZW177) (---) for aminopterin and 5-fluoro-2'-deoxyuridine were the average of six and five separate experiments, respectively. We calculate a linear correlation coefficient of 0.967 and 0.975 for the solid and dashed lines, respectively, in panel A.

strain without the inhibitor are very similar (~2-fold greater) to those reported by Kunkel (16) for a *ung* mutant and a wild-type parent strain. Taken together, these results suggest that the cells expressing the inhibitor protein are at least as defective in uracil-DNA glycosylase activity as their genetically *ung* counterparts. Our findings that expression of the inhibitor gene did not affect the growth rate further suggest that uracil-DNA glycosylase may be a nonessential gene product; however, our experiments do not rule out a secondary cellular function for this enzyme.

Within 4 min after infection of *B. subtilis* with bacteriophage PBS2, the host uracil-DNA glycosylase becomes inactivated (8). Cone et al. (4) purified and partially characterized the phage-induced protein responsible for inhibiting the uracil-DNA glycosylase. Our results suggest that the inhibitor protein is, in fact, a PBS2 gene product. We find that the cloned uracil-DNA glycosylase inhibitor has in vitro properties very similar to those of the inhibitor isolated from PBS2-infected *B. subtilis*. Both are acidic proteins that are extremely heat stable with a molecular weight of about 18,000. Our data confirm that the inhibitor contains a protein component required for activity. Cone et al. (4) have also suggested the possibility that some other molecular component may be part of the inhibitor; however, their data show that the inhibitor was not sensitive to either RNase or DNase and appeared not to be glycosylated. If another modification of the protein exists which is required for activity, it must be capable of being generated in both *B. subtilis* and *E. coli*. In addition to the PBS2 inhibitor, *E. coli* phage T5 also induces a uracil-DNA glycosylase inhibitor (35) although the T5 genome does not become highly substituted with uracil residues like PBS2 DNA.

The properties of the *E. coli* clone that expresses uracil-DNA glycosylase inhibitor described here and those of the *E. coli ung* mutant described by Duncan et al. (6) are very similar. In both cases the lack of glycosylase activity does not alter normal growth rate, it does allow growth of uracil-containing phage, and it confers a weak mutator phenotype. About 10-fold-higher spontaneous mutation frequencies were observed for mutations to nalidixic acid and chloramphenicol resistance by the Ung phenotype. These values are in close agreement with the fivefold increase in mutation frequency to antibiotic resistance reported for the *E. coli ung* mutant (6). Similarly, Duncan and Weiss (7) have shown that G · C → A · T transitions are stimulated about 30-fold in *ung* mutants. The mutagenic consequence of a defective uracil-DNA glycosylase appears to be similar in *S. cerevisiae*. Forward mutation rates of nuclear genes to give resistance to several drugs has been reported to vary from 4 to 40 times that of the wild-type strain (3). These results are consistent with Lindahl's proposal (19) that a physiological function of uracil-DNA glycosylase is to prevent mutagenesis due to deamination of cytosine in DNA.

The action of antifolate drugs limit in vivo levels of thymidylate and bring about rapid loss of cell viability. Treatment of mammalian cells with methotrexate, for example, results in a decrease of the intracellular dTTP pool and dramatic elevation of the dUTP pool, leading to a readily detectable amount of dUMP in DNA (10, 11, 14). Accordingly, the cytotoxicity of folate antagonist has been correlated to the uracil-DNA glycosylase-initiated degradation of cellular DNA as a result of significant dUMP incorporation. This hypothesis was tested by investigating the responses of *E. coli* JM101(pZW177) and JM101(pUC19) to aminopterin. We determined that both the strain that lacked uracil-DNA glycosylase and its isogenic parent exhibited equal sensitivity to survival. Similar results were also reported for the yeast *ung1-1* mutant (3). Assuming that under this condition dUMP is incorporated into cellular DNA, these data suggest either (i) that uracil-DNA glycosylase initiated DNA degra-

dation may not contribute significantly to the onset of cytotoxicity, (ii) that residual uracil-DNA glycosylase activity in the JM101(pZW177) strain may be sufficient to create a cytotoxic effect, or (iii) that another repair mechanism may exist for removing uracil from DNA thus masking the role of uracil-DNA glycosylase in antifolate-effected cytotoxicity. One candidate that might initiate a second type of uracil-DNA repair would be endonuclease V (9). This enzyme acts efficiently on uracil-containing DNA and on a variety of other types of DNA damage, but its physiological role remains unknown. Since inhibiting dihydrofolate reductase may block several metabolic pathways other than dTMP biosynthesis, we also investigated the effect of a more specific inhibitor of dTMP synthesis, 5-fluoro-2′-deoxyuridine, on cytotoxicity. 5-Fluoro-2′-deoxyuridine-induced imbalance of the intracellular dTTP pool and subsequent double-strand breaks in DNA has also been shown to be accompanied by cell death (38). Our results showing that the clone which lacked uracil-DNA glycosylase activity was slightly resistant to 5-fluoro-2′-deoxyuridine was similar to the report for the response of *B. subtilis urg* mutant to thymineless conditions as observed by Makino and Munakata (23). Contrary to our expectations, these data do not seem to support the above hypothesis. Rather they tend to suggest uracil-DNA glycosylase-initiated DNA degradation may not account for the primary cause of cytotoxicity from these drugs.

Uracil-DNA glycosylases have been isolated from a wide variety of organisms. However, the physiological function of this enzyme has only been investigated in only a few cases, including *E. coli* (6, 7), *B. subtillis* (22, 23), *S. cerevisiae* (3), and *Ustilago maydis* (37), where uracil-DNA glycosylase mutants are available. A major problem in studying uracil-DNA repair mechanisms in mammalian cells is the lack of defined genotypic mutants for physiological and biochemical analysis. An alternative approach for obtaining uracil-DNA glycosylase-deficient cells is suggested from our present study. If the cloned PBS2 uracil-DNA glycosylase inhibitor gene were successfully expressed, for example, in a mammalian cell, we anticipate that uracil-DNA glycosylase would become inactive. This prediction is largely based on the in vitro observation by Karran et al. (15) that the inhibitor protein is active against several uracil-DNA glycosylases ranging from bacteria to human but inactive against 3-methyladenine-DNA glycosylase and 2,6-diamino-4-hydroxy-5-(*N*-methylformamido) pyrimidine-DNA glycosylase of *E. coli* or hypoxanthine-DNA glycosylase isolated from calf thymus. Hence, the action of the inhibitor protein seems highly specific. These properties should make the inhibitor a useful tool for elucidating the uracil excision repair pathway and perhaps creating uracil-DNA glycosylase-defective mutants in other cells.

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