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Generation of DNA-Free *Escherichia coli* Cells by 2-Aminopurine Requires Mismatch Repair and Nonmethylated DNA

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Undirected mismatch repair initiated by the incorporation of the base analog 2-aminopurine kills DNA-methylation-deficient *Escherichia coli dam* cells by DNA double-strand breakage. Subsequently, the chromosomal DNA is totally degraded, resulting in DNA-free cells.

The DNA mismatch repair (MMR) system has evolved as an error correction mechanism following DNA replication, but it acts also as an editor of homologous recombination (14). Intriguingly, MMR can kill bacterial and mammalian cells by inactivating cellular DNA bearing mismatches recognized by this DNA repair system (14). Several widely used chemotherapeutic agents kill tumor and normal cells by this mechanism, providing a ground for direct selection of mismatch repair-deficient mutator cells resistant to such treatments and additionally prone to evolution of new cancers (1, 3).

In enterobacteria, such cell killing requires the absence of DNA Dam methylation (adenine methylation at GATC sequences), allowing the MMR machinery to produce lethal double-strand breaks (DSB) (9). Such mismatch-stimulated killing (6) is so efficient that mismatch repair-deficient mutants (*mutS*, *mutL*, and *mutH*) were isolated as survivors of *Escherichia coli dam* mutants treated with the base analogue 2-aminopurine (2-AP) (8). The incorporation of 2-AP in DNA leads to the 2-AP · T and 2-AP · C mispairs that are recognized by the MMR.

The mismatch-stimulated killing requires functional mismatch recognition (MutS), repair-initiating protein matchmaker (MutL), nickase cutting the unmethylated strand of hemimethylated double-stranded GATC sequence (MutH endonuclease), and helicase II (UvrD) proteins (5, 6, 14). Because a single mismatch is sufficient to cause mismatch-stimulated killing (6), a coincident attack by MutH on GATC sequences (flanking the mismatch) on opposite strands, followed by convergent unwinding by helicase II, probably causes the DSB. Even without any exogenous introduction of mismatched bases, the RecABCD-dependent repair of DSBs is required for the viability of *dam* mutants, suggesting that at least one replication error (mismatch) is generated in each replication cycle (8, 13). The viability of the *dam recA mutS* (*mutL* or *mutH*) triple mutants provides the argument that mismatch repair is the potential killer of *dam* mutants also, under normal replication conditions (8, 11).

Here, we have studied the fate of *E. coli dam* cells exposed to the lethal effects of 2-AP. The survival analysis of 2-AP-

TABLE 1. Strains used in this study

Strain	Relevant genotype	Genotype or phenotype	Origin/reference
JJC520	Wild type	*deoA21 lac*$_{624}$ *lacY1* Cm^r	2
MK1	*dam*	As JJC520 but Δ*dam-16*::Kan^r	P1 transduction of Δ*dam-16*::Kan^r from *E. coli* Genetic Stock Center strain no. 7308
MK2	*mutS*	As JJC520 but *mutS* Ω(Sm^r/Sp^r)	P1 transduction of *mutS* Ω(Sm^r/Sp^r)/(4)
MK3	*dam mutS*	As MK1 but Δ*mutS* Ω(Sm^r/Sp^r)	As above
MK4	*recD*	As JJC520 but *recD1901*::Tn*10*	P1 transduction of *recD1901*::Tn*10* from *E. coli* Genetic Stock Center strain no. 7429
MK5	*dam recD*	As MK1 but *recD1901*::Tn*10*	As above
MK6	Wild type	As JJC520 but *mhpC281*::Tn*10* Cm^s	P1 transduction of *mhpC281*::Tn*10* from *E. coli* Genetic Stock Center strain no. 7333
MK7	*dam*	As MK6 but *dam-13*::Tn*9*	P1 transduction of *dam13*::Tn*9* from GM2199 strain kindly provided by M.G. Marinus
MK8	*mutH*	As MK6 but *mutH471*::Tn*5*	P1 transduction of *mutH471*::Tn*5*/(12)
MK9	*dam mutH*	As MK7 but *mutH471*::Tn*5*	As above
MK10	*uvrD*	As MK6 but *uvrD*::*phleo*	P1 transduction of *uvrD*::*phleo*/(16)
MK11	*dam uvrD*	As MK7 but *uvrD*::*phleo*	As above

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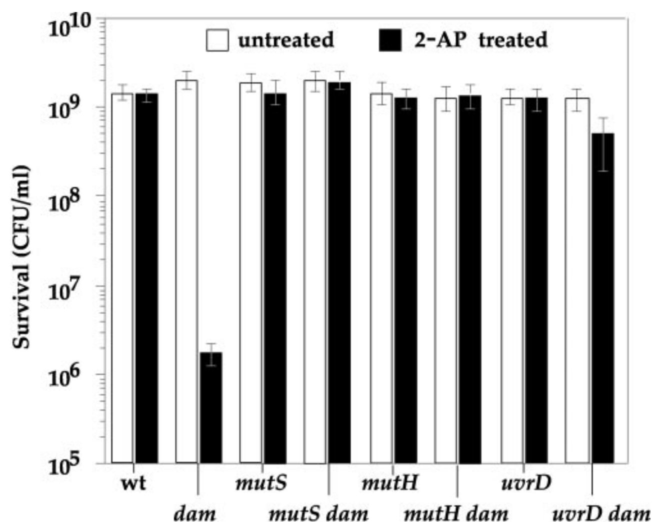

FIG. 1. Survival of 2-AP treated cells. Overnight cultures grown in M9 minimal medium-glucose were diluted and plated on M9 minimal medium-glucose containing (or not containing) 500 μg/ml of 2-AP and incubated at 37°C. Each point represents the mean (± standard error) of six independent experiments. wt, wild type.

treated *dam* cells (Table 1 shows a list of strains) shows that the MutS, MutH, and UvrD proteins are responsible for 10^3-fold viability loss in *dam* cells treated with 2-AP (Fig. 1), which corroborates previously published data (8). Flow cytometry and fluorescent microscopy were used to monitor the cellular DNA content. For the flow cytometry analysis, an overnight culture made in LB was diluted 1,000-fold in LB, and bacteria were grown at 37°C until reaching an optical density at 600 nm of 0.05. The culture was split into two; the first (control) culture continued to grow up to an optical density of 0.4 before addition of 250 μg/ml of rifampin. Rifampin prevents initiation of replication but allows the finalization of the replication cycles. After 90 min of incubation at 37°C, 0.4 ml of culture was withdrawn and mixed with 1.6 ml of cold methanol (100%) and left on ice for 1 h. After centrifugation, the resulting pellet was resuspended in 1.5 ml of 0.01 M Tris (pH 7.4), 0.01 M $MgCl_2$, and DAPI (4′,6′-diamidino-2-phenylindole) (0.3 μg/ml) (15). Flow cytometry was performed with an Epics-ELITE (Coulter). The second test culture was treated with 1 mg/ml of 2-AP for 2 h 30 min and then centrifuged (2 min at room temperature) to eliminate the 2-AP before the rifampin treatment, as described above.

Flow cytometry analysis shows that the 2-AP pretreatment of the wild-type strain produced, besides the two major peaks containing two and four chromosomes, also two small peaks corresponding to one and three chromosomes, indicating induction of significant asynchrony at the DNA replication initiation level or chromosome degradation (Fig. 2). In contrast, in the *dam* mutant, the same treatment resulted in disappearance of all peaks, indicating the loss of the complete set of chromosomes. The only detectable peak was composed of cells deprived of most of their DNA, which was confirmed by the fluorescence microscopy (Fig. 3). The chromosomal degradation in the 2-AP-treated *dam* mutant is dependent on active MutS, MutH, and UvrD proteins (Fig. 2).

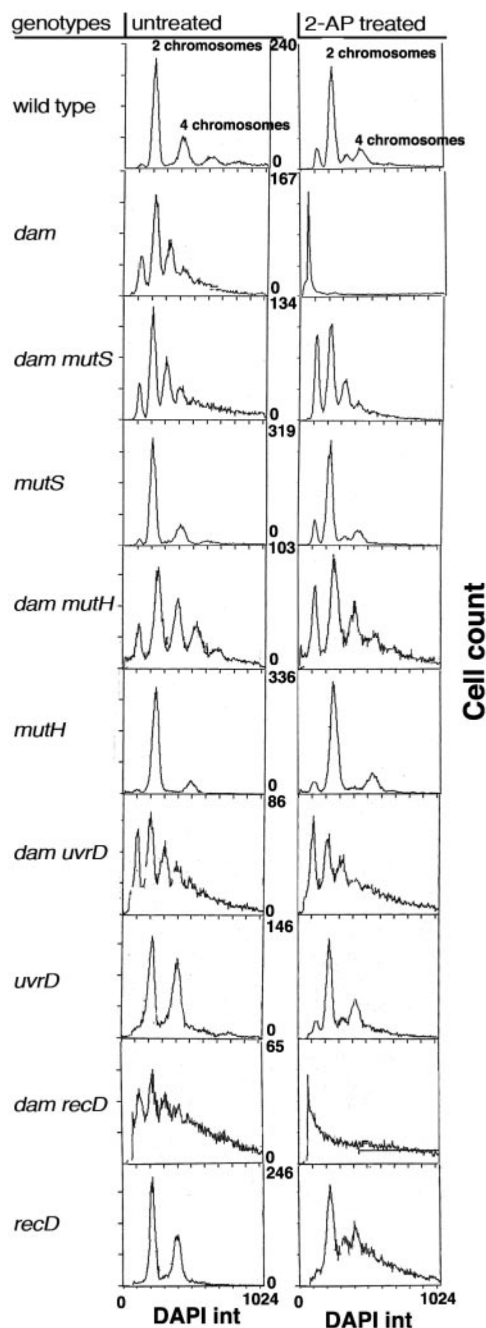

FIG. 2. Flow cytometry analysis of DNA content in different strains with or without 2-AP treatment. DAPI int (integral) is the area signal of the pulses produced at the detector as the cell passes through the measurement region.

As this result was obtained after successive 2-AP and rifampin treatments, we wondered whether 2-AP treatment alone could induce chromosome degradation in *dam* mutants. The flow cytometry analysis of the *dam* cells incubated with 2-AP for various periods showed that the peak of bacteria without DNA started to emerge after 2 h of treatment and it became dominant after 3 h (Fig. 4). Therefore, the rifampin treatment does not participate in DNA degradation. However,

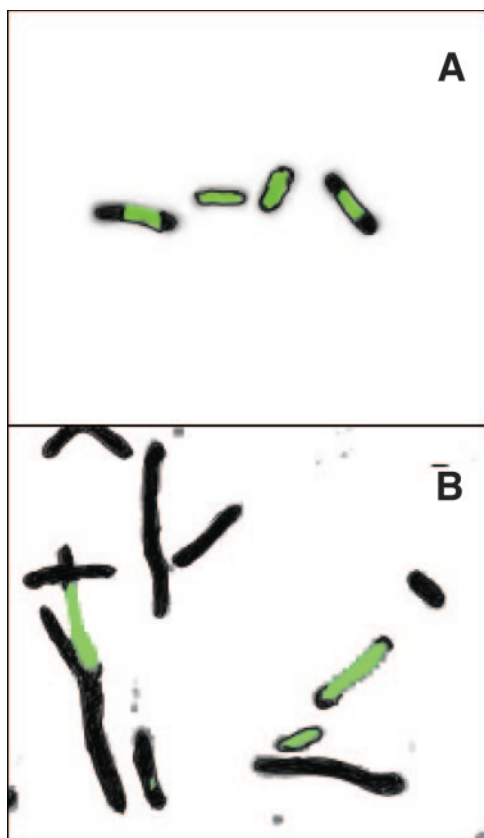

FIG. 3. Production of DNA-free bacteria after 2-AP treatment in the *dam* mutant. The *E. coli dam*-mutant culture was treated with 2-AP as described in the text, and then cells were visualized using phase-contrast and fluorescence microscopy. DNA was stained with DAPI. (A) Untreated cells; (B) 2-AP treated cells. DAPI-stained DNA is green, and DNA-less cell space and DNA-less cells are black.

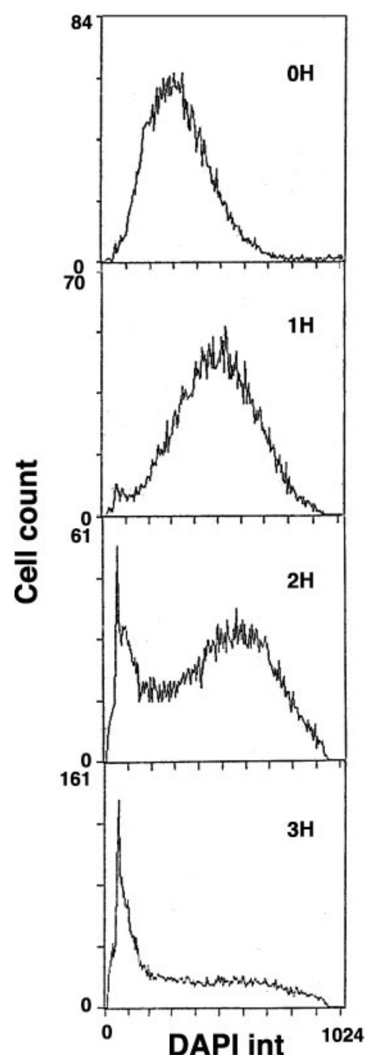

FIG. 4. Kinetics of chromosome degradation in 2-AP-treated *dam* cells without addition of rifampin. *dam*-mutant culture was treated with 2-AP and incubated at 37°C for different times as indicated. Samples were precipitated with methanol and analyzed as described in the text. DAPI int (integral) is the area signal of the pulses produced at the detector as the cell passes through the measurement region.

rifampin affects the kinetics of chromosome degradation (Fig. 5A). This was demonstrated by treating cells for 1 h with 2-AP, which is insufficient to produce the peak of DNA-less bacteria, and by subsequently incubating cells with rifampin. We observed that a 30-min postincubation was sufficient to produce a substantial fraction of DNA-free bacteria, indicating that the addition of rifampin apparently accelerates DNA degradation (Fig. 5A).

In this regimen, we treated bacteria with 2-AP for more than two generations (generation time was 30 min), which might produce DNA with two complementary strands containing 2-AP. Hence, the question arises whether the incorporation of this base analogue into one of the strands is sufficient to trigger degradation of DNA in a *dam* mutant. Fixing the rifampin treatment to 90 min, we varied the time of 2-AP treatment, withdrawing samples, centrifuging them at room temperature for 2 min in order to eliminate the 2-AP, and then resuspending them in LB containing rifampin. Figure 5B shows that the incorporation of the 2-AP for the time span of one generation is largely enough to trigger the chromosome degradation. Even the short exposure of cells to the 2-AP (2 min required for centrifugation) could alter the formation of mature chromosomes (Fig. 5B). This result shows that the incorporation of 2-AP on only one DNA strand is sufficient to trigger DNA degradation.

Finally, as the RecBCD-dependent recombination machinery is required for the viability of *dam* mutants (10, 17), we tested whether extensive chromosome degradation results from its exonuclease V activity. The analysis of the DNA content of 2-AP-treated *dam recD* cells (lacking exonuclease V activity) indicates that degradation depends only partially on exonuclease V activity (Fig. 2). This is in contrast to DNA breakdown initiated by radiation-induced DSBs, where the global degradation depends on RecBCD (7). The MMR-generated DSBs are expected to be different from DSBs induced by radiation or restriction enzymes. Initiated by MutH nicking, on opposite strands, of GATC sequences flanking a 2-AP mis-

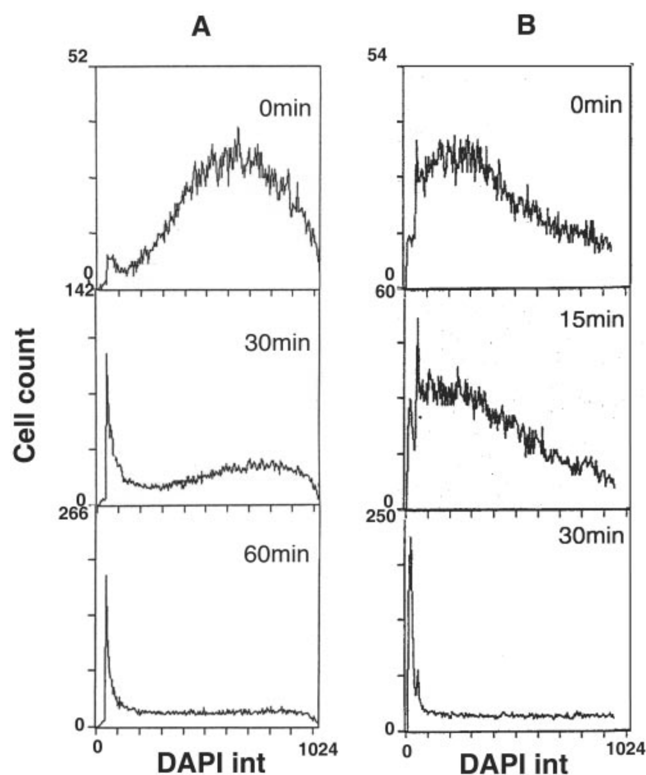

FIG. 5. The effect of variable time of rifampin and 2-AP treatment on chromosome degradation in *dam*-mutant cells. (A) *dam* culture treated with 2-AP for 1 h at 37°C was centrifuged, resuspended, and incubated in LB containing rifampin. At different times, samples were precipitated with methanol and analyzed as described in the text. (B) 2-AP was added to a *dam* culture and, after various times, 0.4 ml of culture was centrifuged for 2 min at room temperature and the pellet resuspended in LB containing rifampin, followed by incubation for 90 min at 37°C before methanol treatment. Finally, cells were analyzed as described in the text. DAPI int (integral) is the area signal of the pulses produced at the detector as the cell passes through the measurement region.

match, the DSB would result from convergent-DNA unwinding by UvrD helicase. The DNA ends with single-stranded tails are better substrates for single-strand exonucleases (e.g., RecJ, ExoI, ExoVII, and ExoX) than for the double-strand exonuclease RecBCD. All these nucleases may alternate in the global DNA breakdown. The described efficiency of producing DNA-free *E. coli* cells offers a facile method for experimentation with bacterial cells devoid of their genes.

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