Genetic Analysis of Repair and Damage Tolerance Mechanisms for DNA-Protein Cross-Links in *Escherichia coli*

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DNA-protein cross-links (DPCs) are unique among DNA lesions in their unusually bulky nature. We have recently shown that nucleotide excision repair (NER) and RecBCD-dependent homologous recombination (HR) collaboratively alleviate the lethal effect of DPCs in *Escherichia coli*. In this study, to gain further insight into the damage-processing mechanism for DPCs, we assessed the sensitivities of a panel of repair-deficient *E. coli* mutants to DPC-inducing agents, including formaldehyde (FA) and 5-azacytidine (azaC). We show here that the damage tolerance mechanism involving HR and subsequent replication restart (RR) provides the most effective means of cell survival against DPCs. Translesion synthesis does not serve as an alternative damage tolerance mechanism for DPCs in cell survival. Elimination of DPCs from the genome relies primarily on NER, which provides a second and moderately effective means of cell survival against DPCs. Interestingly, Cho rather than UvrC seems to be an effective nuclease for the NER of DPCs. Together with the genes responsible for HR, RR, and NER, the mutation of genes involved in several aspects of DNA repair and transactions, such as *recQ, xth nfo, dksA*, and *topA*, rendered cells slightly but significantly sensitive to FA but not azaC, possibly reflecting the complexity of DPCs or cryptic lesions induced by FA. UvrD may have an additional role outside NER, since the *uvrD* mutation conferred a slight azaC sensitivity on cells. Finally, DNA glycosylases mitigate azaC toxicity, independently of the repair of DPCs, presumably by removing 5-azacytosine or its degradation product from the chromosome.

The DNA molecules of living organisms continuously suffer from various types of damage resulting from exposure to endogenous and environmental genotoxic agents. Damage to DNA impairs the faithful propagation of genetic information during replication and transcription, exerting deleterious effects on cells (20). DNA-protein cross-links (DPCs) are unique among DNA lesions in that they are extremely bulky compared to conventional bulky lesions, such as pyrimidine photodimers and the base adducts of aromatic compounds. DPCs are produced by a number of chemical agents, such as aldehydes and heavy metal ions, and also by physical agents such as ionizing radiation and UV light (reviewed in reference 3). DPCs have also been identified in cells or nuclei treated with antitumor agents (4, 10, 44, 62). In addition, we have shown that oxazine, which is produced by nitrosative damage to guanine, mediates the formation of DPCs and polyamine cross-link adducts (49, 50, 52). Thus, understanding the repair and/or damage tolerance mechanism of this ubiquitous and unique class of DNA lesions will provide further insight into how cells maintain genetic integrity and ensure survival in the face of genomic insults. However, the repair and damage tolerance mechanisms of DPCs have long remained elusive, partly because many but not all DPC-inducing agents produce other types of DNA lesions simultaneously, making it rather difficult to elucidate the repair and tolerance mechanisms associated with DPCs alone. Although preceding studies of the sensitivities of repair-deficient *Escherichia coli* mutants to DPC-inducing agents such as formaldehyde (FA) and 5-azacytidine (azaC) provided intriguing insights into the mechanisms of DPC processing in cells (5, 40, 54, 70), a unified mechanism has not yet been established.

We have recently shown in vivo and in vitro evidence that nucleotide excision repair (NER) and homologous recombination (HR) cooperate closely to mitigate the genotoxic effect of DPCs in *E. coli* cells (51). NER removes DPCs with cross-linked proteins smaller than 12 to 14 kDa, whereas oversized DPCs are processed exclusively by RecBCD-dependent HR. The upper size limit of DPCs amenable to NER is determined by the loading efficiency of UvrB, the damage recognition protein in NER, onto DPCs. Consistent with this mechanism, the NER incision efficiency for DNA containing DPCs varies significantly with the size of cross-linked proteins and peptides in vitro (2, 46, 47, 51, 58). Interestingly, no chromosome breakage was observed in cells following FA treatment, although the HR of DPCs proceeded through the RecBCD pathway (51), which is specific to recombination initiated at DNA double-strand breaks. Taken together, these results indicate that *E. coli* cells employ both repair and damage tolerance mechanisms for DPCs. However, a number of repair and damage tolerance genes still remain to be examined for obtaining an entire picture of the repair and tolerance mechanisms of DPCs.
In the present work, we have systematically assessed the sensitivities of E. coli mutants defective in HR, replication restart (RR), translesion synthesis (TLS), NER, base excision repair (BER), transcription, and topological changes of chromosomes to DPC-inducing agents, including FA and azaC. FA is a relatively nonspecific DPC-inducing agent that produces DPCs containing various proteins of sizes greater than 7 kDa in E. coli (51). Thus, FA treatment of E. coli results in chromosomal DPCs that are processed by both NER (small DPCs) and HR (large DPCs). Conversely, azaC is a specific DPC-inducing agent. azaC is metabolized and incorporated into DNA, covalently trapping DNA cytosine methyltransferases (Dcm) (21, 65). Accordingly, azaC treatment of E. coli likely results in chromosomal DPCs containing 5-kDa Dcm, which is the sole Dcm in E. coli K-12. Due to the large size of the Dcm protein, Dcm-DPCs are processed by HR. The present results reveal differential or cryptic roles of repair and miscellaneous genes in the processing of DPCs and provide further insights into how cells survive when chromosomal DNA takes on the burden of unusually bulky lesions. Our data also suggest that DNA glycosylases mitigate azaC toxicity, presumably by removing 5-azacytosine or its degradation product from the chromosome.

MATERIALS AND METHODS

Strains, plasmids, and media. All strains used in this study are derivatives of E. coli K-12 (1, 6–8, 13, 17, 19, 27, 29, 30, 34–37, 48, 53, 60, 63, 67, 68, 71, 72, 76, 78, 79). The relevant genotypes of the strains and the properties of plasmids are listed in Table 1. A portion of the strains in Table 1 was constructed in this study. The strains deficient in list of Table 1. A portion of the strains in Table 1 was constructed in this study.

Cell survival assays with FA and azaC. The working solution of FA was prepared freshly from the 37% FA solution (Nacalai Tesque) for every experiment. azaC (Sigma) was dissolved in 50% acetic acid and stored at −20°C until use. Except for the priA and other related primosomal mutants (priB, priC, and rep), cells were grown to an optical density at 600 nm (OD600) of 0.3 at 37°C in LB medium for FA treatment or in minimal M medium for azaC treatment. A total of 0.2 ml of culture was diluted with 4.6 ml of 66 mM phosphate buffer (pH 6.8) containing different concentrations of FA (indicated in the figures) or with 4.8 ml of minimal M medium containing different concentrations of azaC (indicated in the figures), incubated at 37°C for 30 min with shaking. The priA, priB, priC, and rep mutants were similarly grown and treated with FA and azaC in M9 medium, since the priA mutant is sensitive to rich media (36). After FA or azaC treatment, cells were diluted and plated on M9 agar plates, and colony formation was typically analyzed after overnight incubation at 37°C. Some strains used in this study grew slowly on M9 plates (AQ10459 [wild type (wt)] and AQ10479 [pro4]) or LB plates (RFM445 [gyrB(Ts)] and RFM475 [gyrB(Ts) topA]) (see Table S1 in the supplemental material) so that colony formation was analyzed after a few days of incubation. Cells transformed with pNTR-SD-Dcm (Table 1) were grown to an OD600 of 0.2 and incubated with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 10 min to induce Dcm. The subsequent FA and azaC treatments were performed as described above for cells without a plasmid. Unless otherwise noted, the survival data are based on three to five independent experiments. Statistical significance was determined by a two-sided unpaired Student’s t-test. A P value of less than 0.05 was defined as significant.

RESULTS

RR following HR of DPCs depends on PriA but not Rep helicase. In E. coli, the PriA, PriB, and PriC proteins play vital roles in restarting chromosome replication through two PriA-dependent mechanisms, i.e., the PriA-PriB and PriA-PriC pathways (Fig. 1A) (24, 36). The RR proteins recognize forked DNA, and mutant cells were treated with various concentrations of FA and azaC for 30 min, and the cell survival was analyzed. The priA mutant was hypersensitive to both FA and azaC (Fig. 1B and E), indicating that RR following the HR of DPCs proceeds through PriA-dependent mechanisms. Compared to wt, the priB mutant exhibited a moderate sensitivity to azaC at high concentrations (Fig. 1F), although it was not sensitive to FA (Fig. 1C). The priC mutant also showed a slight sensitivity to azaC at high concentrations (Fig. 1F), but the sensitivity increase was not statistically significant. The differential azaC
addition to the PriA-dependent RR pathways, there is an alternative Rep-PriC restart pathway to load DnaB onto the forked DNA structures (63). However, the rep mutant was sensitive to neither FA nor azaC (Fig. 1C and F), indicating that the Rep-PriC pathway is dispensable in RR following the HR of DPCs.

sensitivities of the priB and priC mutants suggest that the PriA-PriB pathway contributes more to RR than the PriA-PriC pathway does, but the two pathways can compensate for each other to a significant degree when one is compromised. In addition to the PriA-dependent RR pathways, there is an alternative Rep-PriC restart pathway to load DnaB onto the forked DNA structures (63). However, the rep mutant was sensitive to neither FA nor azaC (Fig. 1C and F), indicating that the Rep-PriC pathway is dispensable in RR following the HR of DPCs.

### TABLE 1. Strains and plasmids used in this study

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**Plasmids**

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* NBRP, National BioResource Project; CGSC, Coli Genetic Stock Center.
* b HRS7052 was derived from the strain described in reference 35.
We used M9 as a common medium for the FA treatment of RR mutants (Fig. 1B and C), since one of the RR mutants used (\textit{priA}) is sensitive to rich media (36). Conversely, rich LB medium was used for the FA treatment of the other sets of mutants (Fig. 2 to 5). The use of M9 medium significantly increased the FA sensitivity of cells. For instance, AB1157 (wt) was more sensitive in M9 medium than in LB medium, with the sensitivity in M9 being comparable to that of AQ10459 (wt) in M9 medium (Fig. 1D). Thus, FA concentrations used in the survival assays of RR mutants (0 to 2 mM) (Fig. 1B and C) were lower than those used for other sets of mutants (0 to 7 mM or 0 to 16 mM) (Fig. 2 to 5). In view of the high reactivity of FA to amino and sulfhydryl compounds, it is likely that the constituents of rich LB medium scavenged FA more effectively than those of M9 medium. The azaC sensitivity of RR mutants was also assayed in M9 medium. The azaC concentrations used for the \textit{priB}, \textit{priC}, and \textit{rep} mutants were the same as those used for other sets of mutants (Fig. 2 to 5). However, azaC concentrations used for the \textit{priA} mutant were much lower than those for other sets of mutants due to an extreme sensitivity of the mutant. The \textit{priA} mutant was hypersensitive to both FA and azaC, but the sensitivity to azaC was by far greater than that to FA (Fig. 1B and E). Also, the \textit{priA} mutant grew very slowly (see Table S1 in the supplemental material) and was more sensitive to azaC than the \textit{recA} mutant (Fig. 1E). Taken together, it is likely that the extreme azaC sensitivity of the \textit{priA} mutant exhibiting complex phenotypes is attributable to defects not only in RR but also in yet-unknown factors.

\textbf{Overproduction of Dcm increases azaC sensitivity of cells.}\n
We have previously proposed that DPCs containing large cross-linked proteins (\textgtr12 to 14 kDa) are not repaired by NER and exclusively processed by RecBCD-dependent HR that involves the genes \textit{recA}, \textit{recBCD}, \textit{recG}, and \textit{ruvABC}. We confirmed that these mutants were sensitive to FA, whereas the \textit{recF} mutant was not (see Fig. S2A in the supplemental material). azaC incorporated into the chromosome likely traps the Dcm protein (53 kDa), giving rise to large DPCs (Dcm-DPCs) that are exclusively processed by RecBCD-dependent HR. Consistent with this mechanism, it was reported that overproduction of the Dcm protein from the \textit{dcm}-carrying plasmid increased the azaC sensitivity of wt cells by 930-fold and increased that of the \textit{recA} mutant by fivefold (5). However,
Interestingly, the same report showed that the dcm mutant was not particularly resistant to azaC compared to the corresponding wt cell that expressed a physiological level of Dcm (5). Our tentative interpretation of the apparently inconsistent results regarding the effects of overproduction and mutational inactivation of Dcm is that the HR capacity of the repair-proficient wt cells is sufficient enough to deal with Dcm-DPCs produced from endogenous Dcm. Using the fluorescein isothiocyanate-labeling method (51), we attempted to detect Dcm-DPCs in DNA isolated from azaC-treated cells without success (data not shown), suggesting very low levels of the endogenous Dcm protein and concomitant Dcm-DPCs in cells. To confirm the role of HR in the processing of Dcm-DPCs more clearly, we used cells harboring pNTR-SD-Dcm, which overproduces the Dcm protein upon treatment with IPTG. A similar approach has shown that overproduction of EcoRII Dcm results in the formation of Dcm-DPCs (51). We therefore treated the Dcm-overproducing cells with azaC and monitored their survival using the fluorescein isothiocyanate-labeling method (51). We observed that the survival of the Dcm-overproducing cells was significantly lower than that of the wt cells, indicating that Dcm-DPCs are formed in these cells. This result is consistent with the idea that Dcm-DPCs are processed by HR, and it supports our interpretation that the HR capacity of the repair-proficient wt cells is sufficient enough to deal with Dcm-DPCs produced from endogenous Dcm.

FIG. 2. Effects of Dcm overproduction on the azaC sensitivity of HR mutants and survival of recQ and recJ mutants after treatment with FA and azaC. (A to F) Effects of Dcm overproduction on the azaC sensitivity of HR mutants. AB1157 (wt) and HR mutants (recA, recB, recF, recG, and ruvA) harboring pNTR-SD-Dcm (open circles) or no plasmid (filled circles) were treated with 1 mM IPTG for 10 min, followed by azaC in minimal A medium. HR mutants used were KY1056 (recA), ME8083 (recB), IP10 (recF), C266 (recG), and HRS2300 (ruvA). Standard deviations are indicated with error bars. (G and H) FA and azaC treatment of recQ and recJ. Shown are data for AB1157 (wt) (filled circles) and the recJ (squares), and recQ (triangles) mutants. Cells were treated with FA and azaC in LB and minimal A media, respectively. Data points showing statistically significant differences (P < 0.05) between the wt and the mutant are indicated with asterisks. Note that azaC concentrations are displayed on a logarithmic scale.

FIG. 3. Survival of TLS mutants after treatment with FA and azaC. (A) FA treatment of single mutants. Shown are data for AB1157 (wt) (filled circles) and the polB (squares), dinB (triangles), umuDC (diamonds), and polA (inverted triangles) mutants. (B) FA treatment of mutants defective in multiple polymerases. Shown are data for AB1157 (wt) (filled circles) and the dinB umuDC (squares), polB dinB umuDC (triangles), and polA polB dinB umuDC (inverted triangles) mutants. (C and D) azaC treatment of single mutants and those defective in multiple polymerases, respectively. Symbols represent the same strains as shown in panels A and B, respectively. Cells were treated with FA and azaC in LB and minimal A media, respectively. Except for mutants containing the polA mutation, there were no statistically significant differences in cell survival between the wt and the TLS mutants. Note that azaC concentrations are displayed on a logarithmic scale.
replication arrest of plasmid pBR322 at the canonical methylation site in *E. coli* cells grown in the presence of azaC (38). Overproduction of Dcm from a plasmid dramatically increased the azaC sensitivity of cells that were insensitive (wt and *recF*) or moderately sensitive (*recG*) mutants without a plasmid (Fig. 2A, F, and D). Similarly, overproduction of Dcm slightly increased the azaC sensitivity of *recA*, *recB*, and *navA* mutants that were hypersensitive to azaC without a plasmid (Fig. 2B, C, and E). We infer that the HR of *recA*, *recB*, and *navA* mutants was severely impaired so that Dcm-DPCs produced from endogenous Dcm were sufficient to kill the mutants. Thus, overproduction of Dcm-DPCs in the mutants did not result in a dramatic increase in cell killing.

**RecQ, but not RecJ, is slightly sensitive to FA.** The RecQ helicase and RecJ exonuclease are the components of the RecFOR recombination machinery (39). The *recJ* mutant was sensitive to neither FA nor azaC (Fig. 2G and H). The *recQ* mutant displayed no sensitivity to azaC (Fig. 2H) but was slightly sensitive to FA (Fig. 2G). Although the FA sensitivity of the *recQ* mutant was significant, the phenotype to FA was by far milder than that of the *recB* mutant (see Fig. S2A in the supplemental material and reference 51). Accordingly, this is consistent with our previous observation that DPCs are processed exclusively by RecBCD-dependent HR and not RecFOR-dependent HR (51). In addition to its role in the RecFOR pathway, RecQ is suggested to be involved in SOS DNA repair.

**FIG. 4.** Survival of NER and transcription-related mutants after treatment with FA, azaC, UV light, or MMC. Cells were treated with indicated agents. (A to D) NER mutants derived from AB1157. Shown are the wt (filled circles), *uvrA* (triangles), *uvrB* (diamonds), and *uvrC* (squares). (E to G) NER mutants derived from KMBL1001. Shown are data for the wt (filled circles) and the *uvrC* (squares), *uvrD* (triangles), *cho* (diamonds), and *cho uvrC* (inverted triangles) mutants. (H to J) Transcription-related mutants. Shown are data for MG1655 (wt) (filled circles) and the *mfd* (squares), *greA* (triangles), and *dksA* (diamonds) mutants. Standard deviations are indicated with error bars to show the statistically significant sensitivity differences between mutants shown in panels A, C, and E. Also, data points showing statistically significant differences ($P < 0.05$) between the wt and the mutant are indicated with asterisks in panels F and H. Data in panels D, G, and J are based on one or two experiments.
damage signaling in response to replication fork stalling (26). However, it is not clear whether the weak FA sensitivity of the recQ mutant is related to SOS damage signaling.

TLS provides no alternative damage tolerance pathway to DPCs. The presence of a lesion in template DNA often causes the replicative DNA polymerase to stall at the lesion. E. coli cells possess TLS DNA polymerases (Pol), including Pol II (polB), Pol IV (dinB), and Pol V (umuDC), that can transiently take over from the replicative polymerase to continue synthesis across the lesion, allowing replication to resume (55). With UV-induced lesions, there are conflicting reports on the role of Pol II in RR. Pol II was originally implicated in RR (57), but this possibility was ruled out by a subsequent study (11). Interestingly, Pol V becomes essential for RR in the absence of RecJ and RecQ, although the role of Pol V in RR is modest in wt cells (12). To ask whether TLS polymerases play any significant role in the resumption of replication, and hence contribute to damage tolerance to DPCs with respect to cell survival. In contrast, the polA mutants deficient in Pol I (YG2238 and YG6344) were hypersensitive to both FA and azaC (Fig. 3A to D). These results demonstrate the essential role of Pol I as a repair polymerase both in NER and HR.

NER mutants exhibit various degrees of FA and azaC sensitivity. We have previously shown that the uvrA mutant is sensitive to FA but not azaC, demonstrating that NER partly contributes to the repair of FA-induced DPCs. Here we assessed the roles of a series of NER genes, including uvrA, uvrB, uvrC, uvrD, cho, and mfd (73), and those of repair-related transcription factors, including dksA and greA (72), in the processing of DPCs. We confirmed the moderate UV sensitivity of the mfd mutant (Fig. 4J) (66) and the lack thereof of the cho mutant (Fig. 4G) (48). The mutants were treated with FA and azaC, and their sensitivities were determined.

For analysis of NER genes, we used two sets of mutants derived from AB1157 and KMBL1001. With AB1157 derivatives, the mutants involved in damage recognition (uvrA and uvrB) and the dual incision of DNA (uvrC) were sensitive to FA, but their sensitivities differed significantly from each other (uvrB > uvrA > uvrC) (Fig. 4A). The mutants were also sensitive to UV and MMC (Fig. 4C and D), and shared a common order of sensitivity to FA, UV, and MMC (uvrB > uvrA > uvrC). However, the uvrC mutant exhibited a uniquely weak sensitivity to FA but not UV and MMC. The uniquely weak FA sensitivity characteristic of the uvrC mutant was confirmed.
using another set of strains derived from KMB1001 (Fig. 4E). Conversely, the cho mutant exhibited a moderate sensitivity to FA (Fig. 4E), suggesting its in vivo role as an alternative nuclease in the NER of DPCs (see Discussion). To our knowledge, this is the first report of the phenotype of the cho single mutant to DNA-damaging agents. The cho uvrC double mutant exhibited an FA sensitivity comparable to that of the cho single mutant (Fig. 4E). Consistent with its role in NER, the uvrD mutant was sensitive to FA (Fig. 4E), indicating that the UvrD helicase can unwind the duplex containing DPC and dissociate the DPC-containing fragment from its complementary strand. Unlike uvrA, uvrB, uvrC, and cho mutants that were not sensitive to azaC (Fig. 4B and F), the uvrD mutant displayed a slight but statistically significant sensitivity to azaC as well (Fig. 4F), suggesting a role of UvrD outside NER (see Discussion). Mfd is a transcription-coupled repair (TCR) factor that translocates RNA polymerase stalled at the lesion, recruiting UvrA to the site (66, 73). However, the mfd mutant exhibited no sensitivity to FA and azaC (Fig. 4H and I), indicating that unlike the pyrimidine photodimers that are repaired through both global genome repair and TCR pathways, DPCs are eliminated from the genome by global genome repair-NER exclusively.

In E. coli, it has been shown that the backed-up arrays of stalled transcription complexes which are impediments to replication are kept under surveillance of the stringent response regulators ppGpp and DksA or the GreA and Mfd proteins, which revive or dislodge stalled transcription complexes (72). Thus, it would be interesting to determine whether transcription complexes stalled by DPCs are under such a surveillance system. To clarify this, the sensitivities of the dksA and greA mutants to FA and azaC were analyzed. Unlike the mfd mutant, the dksA and greA mutants exhibited no UV sensitivity (Fig. 4I). The greA mutant was not sensitive to FA (Fig. 4H). However, the dksA mutant exhibited a slight but significant sensitivity to FA (Fig. 4H), implying a certain effect of DksA on the stability of transcription elongation complexes trapped by DPCs. The greA and dksA mutants were not sensitive to azaC (Fig. 4I).

**BER mutants are slightly sensitive to FA, and DNA glycosylases alleviate azaC toxicity to cells.** In BER, aberrant bases with minor modifications are removed by DNA glycosylases, and the resulting abasic sites (or nicked abasic sites) are processed by apurinic/apyrimidinic (AP) endonucleases. In E. coli, endonucleases III (nth) and VIII (nei) and formamidopyrimidine glycosylase (fpq) account for the major DNA glycosylase activity for oxidized or fragmented pyrimidines (nth and nei) and purines (fpq) (77). Exonuclease III (nth) and endonuclease IV (nfo) account for the major AP endonuclease activity (16). To clarify whether BER contributes to the repair of DPCs, the sensitivities of glycosylase and AP endonuclease mutants to FA and azaC were assayed. With FA treatment, the nei nth fpq triple mutant and the nth nfo double mutant exhibited a slight but significant FA sensitivity (Fig. 5A and C). The fpq and nei nth mutants were virtually insensitive to FA (Fig. 5A). Thus, elimination of three major DNA glycosylases (or AP lyase activity associated with DNA glycosylases) or two major AP endonucleases confers some FA sensitivity on cells. It remains to be seen whether these BER enzymes are involved in the repair of the cryptic base damage induced by FA (i.e., minor base modifications) or FA-induced DPCs per se, although the latter seems unlikely. However, the relative weak sensitivities of the nei nth fpq triple mutant and the nth nfo double mutant indicate that BER plays at most a minor role in cell survival following FA treatment. Surprisingly, the nei nth fpq triple mutant exhibited unexpected strong sensitivity to azaC (Fig. 5B). However, the azaC sensitivity of the triple mutant was not as high as that of the hypersensitive recA mutant. The fpq single and nei nth double mutants were virtually insensitive to azaC. Also, the nth nfo double mutant was not sensitive to azaC (Fig. 5D). These results imply that Nei/Nth and Fpg glycosylases complement each other in vivo and remove lethal or potentially lethal DNA damage and that Xth and Nfo that generally act following glycosylases in BER are dispensable in mitigating azaC toxicity to cells (see Discussion).

**topI mutants are slightly sensitive to FA.** Not much is known about whether DNA topoisomerases participate in DNA repair in E. coli (39, 69). E. coli has two type I topoisomerases (Topo I and III) and two type II topoisomerases (gyrase and Topo IV), and only Topo III is dispensable for cell growth (9). To obtain insight into the role of topoisomerases in the repair/tolerance of DPCs, we used two Topo mutants (Table 1). RFM445 contains mutations (gyrB221 and gyrB203) in the gyrB gene encoding the gyrB subunit B. The mutations confer coumermycin resistance (Cou r) (gyrB221) and cause temperature sensitivity (gyrB203) (17, 45). The gyrB203 mutation compensates for the lack of DNA relaxing activity associated with the topA mutation (15, 56). Although the gyrB203 allele retains minimum activity to allow cell growth at 37°C, it regains gyrB activity at 30°C and is no longer sufficient as a compensatory mutation, rendering the gyrB203 topA mutant cold sensitive (17). This phenotype was confirmed in the present study (see Fig. S1B in the supplemental material). Keeping in mind the phenotypes described above, we assessed the sensitivity of RFM445 [gyrB(Ts)] and RFM475 [gyrB(Ts) topA] to FA and azaC at 37°C. The gyrB(Ts) mutant was sensitive to neither azaC nor azaC (Fig. 5F and H). However, the gyrB(Ts) topA mutant was slightly sensitive to FA (Fig. 5E), suggesting a role of Topo I in the processing of FA-induced DPCs. The gyrB(Ts) topA mutant was not sensitive to azaC (Fig. 5F).

**General characteristics of genes that alleviate the detrimental effect of DPCs.** Figure 6 shows the summary of the sensitivities to the DPC-inducing agents (FA and azaC) displayed by the mutants, including those deficient in damage tolerance (HR, RR, and TLS), excision repair (NER and BER), and miscellaneous aspects of DNA transactions. The data were derived from those shown in Fig. 1 to 5 and Fig. S2 in the supplemental material. The fold increases in the sensitivities of mutants relative to the corresponding wt were calculated at the FA and azaC concentrations indicated in Fig. 6. With hypersensitive mutants, the survival data at lower concentrations were used for calculation (Fig. 6). Although the data in Fig. 6

**Figure 6 shows the summary of the sensitivities to the DPC-inducing agents (FA and azaC) displayed by the mutants, including those deficient in damage tolerance (HR, RR, and TLS), excision repair (NER and BER), and miscellaneous aspects of DNA transactions. The data were derived from those shown in Fig. 1 to 5 and Fig. S2 in the supplemental material. The fold increases in the sensitivities of mutants relative to the corresponding wt were calculated at the FA and azaC concentrations indicated in Fig. 6. With hypersensitive mutants, the survival data at lower concentrations were used for calculation (Fig. 6). Although the data in Fig. 6
allow only semiquantitative analysis, they reveal general aspects of genes that alleviate the detrimental effect of DPCs (tentative threshold for significance set as a twofold increase in sensitivity). First of all, the damage tolerance mechanism involving HR and subsequent RR provides the most effective means for cell survival against DPCs. TLS does not serve as an alternative damage tolerance mechanism for DPCs so far as cell survival is concerned. Elimination of DPCs from the genome relies primarily on NER, which provides a second and moderately effective means for cell survival against DPCs. Interestingly, Cho rather than UvrC seems to be an effective nuclease for the NER of DPCs. The role of DNA polymerase I (polA) in both HR and NER has been confirmed. Together with the genes responsible for HR, RR, and NER, the mutation of genes involved in several aspects of DNA repair or transactions (i.e., recQ, nei nth fpg, xth nfo, dksA, and topA) rendered cell sensitivities to FA to increase by twofold or slightly more. Except for the nei nth fpg triple mutant, this was characteristic of FA but not azaC, probably reflecting the unique complexity of DPCs induced by FA or other FA-induced cryptic base modifications. UvrD may have a role outside NER, since the uvrD mutation conferred a threefold increase in azaC sensitivity on cells. The triple mutant of DNA glycosylases (nei nth fpg) exhibited weak and moderate sensitivities to FA and azaC, respectively. The moderate azaC sensitivity of the mutant may be related to the removal of azaC incorporated into DNA or related degradation products.

We measured the doubling time of strains used in this study to see whether their growth properties had something to do with FA and azaC sensitivities. Among the strains used, the following strains grew poorly and had more than 1.2-fold-greater doubling time than the wt: priA, recB, nei nth, nei nth fpg, polA, polA dinB polB umuDC, gyrB(Ts), and gyrB(Ts) topA (see Table S1 in the supplemental material). The poorly growing strains exhibited various degrees of FA and azaC sensitivities (see Table S1 in the supplemental material). The mutants

FIG. 6. Comparison of sensitivities of mutants to FA and azaC. White bars, FA sensitivity; black bars, azaC sensitivity. The fold increase in the sensitivity of a mutant relative to the corresponding wt was calculated at the indicated concentrations of FA and azaC. Note that with hypersensitive mutants, the survival data at lower concentrations were used for calculation, since survival data at standard FA and azaC concentrations were not available. The survival data were derived from those shown in Fig. 1 to 5 and Fig. S2 in the supplemental material. The mutants which exhibited more than twofold-greater sensitivities (indicated with an arrow) are indicated by plus signs, and those which did not are indicated by minus signs below the graph. Genes are categorized according to damage tolerance (HR, RR, and TLS), excision repair (NER and BER), and miscellaneous (Misc.) mechanisms. The data under the protease are for the mutant deficient in all cytosolic ATP-dependent proteases (lon, clpAP, clpXP, and hslVU with a sulA background) that was shown not to be involved in DPC processing prior to NER (51).
such as priA, recB, and polA mutants that grow poorly and exhibit low viability under normal conditions may be sensitive to DNA damage for indirect as well as direct reasons.

**DISCUSSION**

In the present study, we examined the FA and azaC sensitivities of a panel of *E. coli* repair mutants to extend our understanding of the repair and tolerance mechanisms of DPCs. The present results have confirmed that HR-dependent damage tolerance and NER-dependent damage repair mechanisms play pivotal roles in cell survival when the genome becomes burdened with DPCs, with the former making a more significant and crucial contribution. The RR following the HR of DPCs relies on the PriA-dependent mechanisms, where the PriA-PriB pathway likely contributes to RR more than the PriA-PriC pathway does, although the two pathways can compensate for each other to a significant degree when one is compromised. Neither TLS nor TCR, a subpathway of NER, is involved in the damage tolerance and repair mechanisms of DPCs.

Recently, the DNA damage response to FA was assessed using chicken DT40 cells with targeted mutations in various DNA repair genes (59). The DT40 cells deficient in HR and TLS were hypersensitive to FA, and those deficient in NER and BER were moderately to slightly sensitive. Thus, the roles of HR, NER, and BER in DPC tolerance and repair are essentially parallel in *E. coli* and DT40 cells, but there is a sharp contrast between the involvement of TLS in *E. coli* and DT40 cells in terms of DPC tolerance. The TLS mutants of DT40 examined for FA sensitivity were REV1, REV3, and RAD18 (59). REV3 is the catalytic subunit of Pol ζ, and REV1 has dCMP transferase activity and may serve as a scaffolding protein which associates with TLS polymerases. RAD18, together with RAD6, forms an E2-E3 complex that monoubiquititates PCNA, likely assisting the switch from replicative to bypass polymerases at the lesion (22). We suspect it is very unlikely that prokaryotic and eukaryotic TLS polymerases directly bypass DPCs in view of the enormous steric hindrance conferred by DPCs, raising the possibility that TLS polymerases have a role outside the direct bypass of DPCs. Furthermore, direct damage bypass is not relevant when DPCs impede the progression of the replicative helicase working ahead of polymerase. It has been suggested that *E. coli* TLS polymerases are involved in RR (12) or template switching after regression of the stalled fork (23), allowing indirect damage bypass. However, inactivation of all of the TLS polymerases (encoded by *polB, dinB*, and *umuDC*) in *E. coli* had no impact on cell survival to FA and azaC (Fig. 3B and D), ruling out this possibility. Functions of TLS polymerases in eukaryotic cells have been suggested to be not only in TLS but also in HR (reviewed in reference 22). Thus, the FA sensitivities of DT40 REV1 and REV3 mutants may be related to HR. It will be interesting to elucidate whether TLS mutants of other eukaryotic cells, such as yeast and mammalian cells, share a similar sensitivity to DPC-inducing agents, as observed in DT40 cells.

In this study, it was suggested that Cho, rather than UvrC, is an effective nuclease for the NER of DPCs, although both cho and *uvrC* single mutants were less FA sensitive than the *uvrA*, *uvrB*, and *uvrD* mutants (Fig. 4A and E). Cho has been implicated in NER and has unique properties, as follows (48). Cho shares significant homology with the N-terminal half of UvrC, which is responsible for incisions at the 3′ side of the lesion. The *cho* mutation does not confer UV sensitivity on cells, but it does slightly increase the sensitivity of the *uvrC* mutant, although we did not observe such an increase in this study (Fig. 4G). Some synthetic bulky lesions that are poorly incised by UvrC are efficiently incised by Cho in vitro. Cho produces only 3′ incisions, and the incision site is four nucleotides further away from the lesion than that of UvrC. Thus, unusually bulky lesions might sterically hinder access by UvrC, but not Cho, to produce the 3′ incision (48, 74). Accordingly, Cho may be able to incise DNA on the 3′ side of DPCs containing proteins of up to a certain size. The 5′ side of DPC would be incised by the C-terminal half of UvrC, which is sufficient to produce the second incision. Interestingly, the additional *uvrC* mutation in the *cho* mutant did not enhance the FA sensitivity (Fig. 4E). A possible interpretation of this result is that UvrC and Cho collaborate with each other in vivo and expand the capacity of NER for DPCs. However, the present result argues against this mechanism, since the *cho* single mutation conferred greater FA sensitivity on cells than the *uvrC* single mutation did, suggesting that the single 3′ incision of DPCs by Cho, but not the 3′ and 5′ dual incisions by UvrC or the combination of Cho and UvrC, is the dominant repair pathway of DPCs. Further biochemical and in vivo studies are necessary to clarify whether this is the case.

Together with mutations in the genes responsible for HR, RR, and NER, we found that those involved in several aspects of DNA repair or transactions conferred cells’ slight but significant sensitivity to FA and/or azaC. *UvrD* is a highly conserved 3′ to 5′ helicase involved in NER and mismatch repair (43, 73). The *uvrD* mutant was sensitive to FA (Fig. 4E), in keeping with our previous result that FA-induced DPCs containing small cross-linked proteins were repaired by NER in vivo (51). In addition, the *uvrD* mutant showed a slight but significant sensitivity to azaC compared to wt and other *uvr* mutant cells (Fig. 4E and F). In view of the size limit of cross-linked proteins amenable to NER (12 to 14 kDa), the DPC containing 53-kDa Dcm induced by azaC should be processed by HR. It has been proposed that UvrD prevents the unnecessary recombination by dismantling the RecA-DNA complex, which may be lethal (18, 75). Accordingly, the UvrD helicase may prevent a deleterious recombination at the replication fork arrested by DPC and thereby mitigate the lethal effect of DPC. However, the contribution of this mechanism to cell survival seems to be moderate, as judged from the mild sensitivity of the *uvrD* mutant to azaC.

In *E. coli*, the supercoiling of chromosomes is regulated by DNA topoisomerases. DNA gyrase (*gyrA* and *gyrB*) introduces negative supercoils, whereas Topo I (*topA*) and Topo IV (*parC* and *parE*) remove excess negative supercoils (9). The *topA* mutant is sensitive to UV light and methanesulfonate (69). Furthermore, topoisomerases are implicated in the HR of DNA damage (39). The *gyrB* mutant partially defective in DNA gyrase was sensitive to neither FA nor azaC (Fig. 5E and F). However, the mutation in the *topA* gene encoding Topo I rendered cells slightly sensitive to FA but not azaC (Fig. 5E and F). The *topA* mutant used here carried an additional *gyrB* mutation as an inevitable compensatory mutation, but
the gyrB(Ts) mutation alone had no impact on cell survival following FA treatment (Fig. 5E). The FA sensitivity of the gyrB(Ts) mutant is not due to redundant activity, since Topo I (topA) and DNA gyrase (gyrB) introduce opposite polarities of supercoils. In the previous study, we demonstrated that FA induces two types of DPCs. One contains proteins covalently trapped on the DNA strand. The other contains proteins covalently bridging two duplex DNA strands. The former is common for FA- and azaC-induced DPCs, but the latter is characteristic of FA-induced DPCs. FA also induces direct interstrand cross-links between DNA bases within a duplex (31). Although similar interstrand cross-links mediated by protein have not been identified so far in FA-treated cells, such cross-links may also be formed by FA. Thus, it is tempting to speculate that the direct or protein-mediated DNA-DNA cross-links may hamper the topological changes of DNA catalyzed by Topo I in HR or NER. The mutants that exhibited damage sensitivity similar to that of topA (i.e., recQ, xth nfo, and dksA mutants) may also be defective in the processing of such DNA-DNA cross-links.

Finally, we found that the nei nth fpg triple mutant, but not the fpg single and nei nth double mutants, was moderately sensitive to azaC (Fig. 5B). Curiously, xth and nfo that act following glycosylases in BER were dispensable in mitigating azaC toxicity (Fig. 5D). These results at least point to the fact that Nei/Nth and Fpg glycosylases complement each other in vivo and remove lethal or potentially lethal DNA damage, although it is not clear why Xth and Nfo are not involved in the subsequent step of BER. In view of the restricted space of the active site pocket of DNA glycosylases that accommodate only minor base modifications (28), it is very unlikely that Nei, Nth, and Fpg recognize extremely large DPCs as damage and excise them from DNA. There seem to be three mechanisms that might account for the azaC sensitivity of the nei nth fpg triple mutant. First, Nei/Nth and Fpg glycosylases are present in the glycosylation and excision of the damaged 5-azaC residues. Second, Nei/Nth and Fpg are involved in the repair of the degradation products of 5-azaC, since 5-azaC is a stable and slow hydrolyzes to ring fragmentation products (42). Like other ring fragmentation products of DNA bases (32, 33), those of 5-azaC left unrepaird in the triple mutant will arrest DNA replication and thereby increase the azaC sensitivity of the cell. Third, in the triple mutant, the AP lyase of Nei, Nth, and Fpg is also inactivated, pointing to a mechanism associated with the reduced AP lyase activity of cells. Analyses of the formation and repair of 5-azaC, degradation products, and Dcm-DPCs in vivo, together with those of the activity of Nei, Nth, and Fpg to 5-azaC and degradation products in vitro, will shed light on the molecular mechanism of azaC toxicity associated with the defect in DNA glycosylases.

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