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*, *dkaA*, 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 oxanine, 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 mecha-

nisms 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.

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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 53-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 *recQ* (NKJ1514) and *recJ* (NKJ1515) were made via P1 transduction of *recQ*::Tn3 from KD2250 (53) and *recJ*::Tn10 from BIK814 (71) into the AB1157 recipient strain, respectively. Transductants were selected for ampicillin resistance (Amp^r) with NKJ1514 and for tetracycline resistance (Tet^r) with NKJ1515. To construct NKJ1500 (*uvrC*), *uvrC*279::Tn10 from CAG12156 (68) was transduced into AB1157 using P1 phage, and UV-sensitive and Tet^r derivatives were selected. Strains YG2238 (*polA*) and YG6341 (*polB*) were obtained by P1 transduction using AB1157 as the recipient and HRS7052 (chloramphenicol resistant [Cam^r]) (35, 76) and HRS6700 (kanamycin resistant [Kan^r]) (67) as donors, respectively. The *dinB* gene was disrupted as an in-frame deletion without a selection marker as described previously (14). Briefly, using the primers *dinB* W-F (5'-CAAACCTGAAATCACTGTACTTT ACCAGTGTGAGAGGTGAGCAATGATTCCGGGGATCCGTCGACC-3') and *dinB* W-R (5'-GCACACCAGAATATACATAATAGTATACATCATAATC CCAGCACCAGTTGTGTAGGCTGGAGCTGCTTCG-3'), the Kan^r cassette on pKD13 was amplified and the resultant fragment was flanked upstream and downstream of the *dinB* gene. The fragment was introduced into AB1157 harboring pKD46, and then recombination events generated Kan^r colonies, which are expected to carry Δ *dinB*::Kan^r. Cultivation of the colonies at 43°C cured the temperature-sensitive (Ts) plasmid pKD46, which had been introduced to allow AB1157 to keep the linear PCR fragments intact with its encoding genes derived from lambda phage. The Δ *dinB*::Kan^r strain without pKD46 was designated YG6158. After introducing another Ts plasmid (pCP20) into YG6158, the strain was incubated at 43°C again. The high temperature induced a recombinase FLP and also removed the Ts plasmid from the strain. The FLP specifically acts on the FLP recombination target (FRT) sequences which had been introduced between the *dinB* flanking regions and the Kan^r cassette in advance (Table 1). The recombination removed the Kan^r cassette and produced the Δ *dinB* strain (YG6162). To construct YG6168, Δ (*umuDC*)596::ermGT was transferred to AB1157 using the DE2302 and EC8 strains as described previously for two-step P1 transduction (19, 78). YG6171 (a *dinB umuDC* double mutant) was constructed by introduction of Δ *dinB*::Kan^r into YG6168, followed by removal of the Kan^r cassette as described above. To introduce Δ *polB*::Kan^r into YG6171, P1 transduction was carried out with HRS6700 as the donor. The resultant *polB dinB umuDC* triple mutant was designated YG6342. Finally, YG6344, which has deletions of *polA*, *polB*, *dinB*, and *umuDC*, was constructed by P1 transduction using YG6342 as the recipient and HRS7052 as the donor. RFM445 [*gyrB203*(Ts)] and RFM475 [*gyrB203*(Ts) Δ (*topA cysB*)204] are the Ts mutants

of topoisomerases. In keeping with the reported Ts properties (17), RFM445 and RFM475 exhibited heat and cold sensitivities, respectively (see Fig. S1 in the supplemental material). pNTR-SD is a set of mobile plasmid clones of *E. coli* open reading frames, and the expression of the open reading frames is strictly controlled by *P_{tac}* and *lacI^q* (61). pNTR-SD containing the *dcm* gene was designated pNTR-SD-Dcm (Table 1).

For FA and azaC treatment, cells were grown in LB, minimal A, or M9 medium. Minimal A medium was comprised of 60 mM K₂HPO₄, 33 mM KH₂PO₄, 7.5 mM (NH₄)₂SO₄, 1.7 mM sodium citrate dihydrate, 1 mM MgSO₄ · 7H₂O, and 0.2% glucose, and supplemented with 1 µg/ml thiamine and 0.2% Casamino Acids (5). The composition of M9 medium was 47 mM Na₂HPO₄ · 12H₂O, 22 mM KH₂PO₄, 8.6 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, and 0.4% glucose, supplemented with 2 µg/ml thiamine, 20 µg/ml thymine, and 100 µg/ml each of arginine, leucine, tryptophan, histidine, and proline (36).

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 A medium for azaC treatment. A total of 0.2 ml of cell culture was diluted with 4.8 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 A 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 LB, minimal A, or 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 [*priA*]] 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-1-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 *t* test. A *P* value of less than 0.05 was defined as significant.

Measurement of UV and MMC sensitivity. For the measurement of UV sensitivity, cells were grown to an OD₆₀₀ of 0.3 at 37°C in LB medium, serially diluted and plated on LB agar plates, and exposed to the UV light at doses indicated in the figures. After overnight incubation at 37°C, the number of growing colonies was counted. The sensitivity to mitomycin C (MMC; Sigma) was measured as described above for FA.

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 structures, such as arrested replication forks and D-loops, and sequentially load the replicative helicase DnaB (41, 64). To determine the roles of PriA, PriB, and PriC in RR following the HR of DPCs, the sensitivities of *priA*, *priB*, and *priC* mutants to DPC-inducing agents were assayed. The wt 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

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or description	Source; reference(s) ^a
Strains		
AB1157	Wild type	K. Yamamoto; 79
YG2238	AB1157 Δ Klenow::cat	T. Nohmi; 76
YG6341	AB1157 Δ polB::kan	This study
YG6158	AB1157 Δ dinB::kan	This study
YG6162	AB1157 Δ dinB	This study
YG6168	AB1157 Δ (umuDC)596::ermGT	This study
YG6171	AB1157 Δ dinB Δ (umuDC)596::ermGT	This study
YG6342	YG6168 Δ dinB Δ polB::kan	This study
YG6344	YG6342 Δ Klenow::cat	This study
NKJ1514	AB1157 <i>recQ</i> ::Tn3	This study
NKJ1515	AB1157 <i>recJ</i> ::Tn10	This study
KY1056	AB1157 <i>recA56 srlC300</i> ::Tn10	K. Yamamoto; 79
ME8083	AB1157 <i>recB21</i>	NBRP; 30
IP10	AB1157 <i>recF</i> ::tet	M. Bichara; 6
C266	AB1157 <i>recG258</i> ::kan	M. G. Marinus; 37
HRS2300	AB1157 <i>nuvA100</i> ::cat	T. Hishida; 27
HRS1200	AB1157 Δ ruvC200::kan	T. Hishida; 34
KY1836	AB1157 <i>uvrA6</i>	K. Yamamoto; 1
AB1885	AB1157 <i>uvrB5</i>	K. Yamamoto; 29
NKJ1500	AB1157 <i>uvrC279</i> ::Tn10	This study
RPC501	AB1157 Δ (<i>xthA-pncA</i>) <i>nfo-1</i> ::kan	S. Yonei; 13
BH20	AB1157 <i>fpg-1</i> ::kan	K. Yamamoto; 8
NKJ1004	AB1157 <i>nth</i> ::cat Δ <i>nei</i> ::kan	K. Yamamoto; 60
KL16	Wild type (BW35)	S. S. Wallace; 7
KL16 <i>nei nth fpg</i>	KL16 <i>nei</i> ::cat <i>nth</i> ::kan <i>fpg</i> ::amp	S. S. Wallace; 7
AQ10459	Wild type	NBRP; 36
AQ10479	AQ10459 <i>priA2</i> ::kan	NBRP; 36
DM4000	Wild type	S. J. Sandler; 63
JC19266	DM4000 Δ (<i>priB</i>)302	S. J. Sandler; 63
JC19165	DM4000 <i>priC303</i> ::kan	S. J. Sandler; 63
JJC213	JJC40 <i>rep</i> ::kan	S. J. Sandler; 63
MG1655	Wild type	R. G. Lloyd; 72
N4879	MG1655 Δ <i>mfd</i> ::kan	R. G. Lloyd; 72
N5301	MG1655 <i>greA</i> ::cat	R. G. Lloyd; 72
N5753	MG1655 Δ <i>dkA</i> ::apra	R. G. Lloyd; 72
KMBL1001	F ⁻ derivative of W1485	N. Goosen; 48
CS5541	KMBL1001 Δ <i>cho</i> (FRT)	N. Goosen; 48
CS5430	KMBL1001 Δ <i>uvrC</i> ::cat	N. Goosen; 48
CS5550	KMBL1001 Δ <i>cho</i> (FRT) Δ <i>uvrC</i> ::cat	N. Goosen; 48
CS5431	KMBL1001 Δ <i>uvrD</i> ::tet	N. Goosen; 48
RFM443	Wild type	M. Drolet; 17
RFM445	RFM443 <i>gyrB221</i> (Cou ^r) <i>gyrB203</i> (Ts)	M. Drolet; 17
RFM475	RFM443 <i>gyrB221</i> (Cou ^r) <i>gyrB203</i> (Ts) Δ (<i>topA cysB</i>)204	M. Drolet; 17
BIK814	<i>recJ</i> ::Tn10	K. Yamamoto; 71
KD2250	<i>recQ</i> ::Tn3	K. Yamamoto; 53
HRS7052 ^b	Δ Klenow::cat	H. Iwasaki; 35, 76
HRS6700	Δ polB::kan	H. Iwasaki; 67
DE2302	Δ (umuDC)595::cat <i>fadR615</i> ::Tn10 <i>purB58</i>	R. Woodgate; 78
EC8	Δ (umuDC)596::ermGT <i>fadR purB</i> ⁺	R. Woodgate; 19
CAG12156	<i>uvrC279</i> ::Tn10	K. Yamamoto; 68
Plasmids		
pKD13	Encodes Kan ^r cassette flanked by FRT sequences, temperature sensitive; Kan ^r Amp ^r	CGSC
pKD46	Encodes the lambda Red system under the control of arabinose-promoter, temperature sensitive; Amp ^r	CGSC
pCP20	Encodes a recombinase, FLP, temperature sensitive; Amp ^r Cam ^r	CGSC
pNTR-SD-Dcm	pNTR-SD containing the IPTG-inducible <i>dcm</i> gene	NBRP; 61

^a NBRP, National BioResource Project; CGSC, Coli Genetic Stock Center.^b HRS7052 was derived from the strain described in reference 35.

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 al-

ternative 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.

FIG. 1. Survival of RR mutants after treatment with FA and azaC. (A) Three RR pathways in *E. coli*. Two pathways are PriA dependent and the other is PriA independent and requires the Rep helicase. (B) FA treatment. Shown are AQ10459 (wt) (filled circles) and *priA* (squares). (C) FA treatment. Shown are data for DM4000 (wt) (filled circles) and the *priB* (squares), *priC* (triangles), and *rep* (diamonds) mutants. (D) Comparison of FA sensitivities of AB1157 and AQ10459 in LB and M9 media. Shown are data for AB1157 in LB (circles) and M9 (squares) media and AQ10459 in M9 (triangles) medium. Data for AQ10459 were taken from that shown in panel B, and those for AB1157 are based on one experiment. (E and F) azaC treatment. Symbols represent the same strains as shown in panels B and C, respectively. For a comparison, the survival curve of *recA* (data taken from that shown in Fig. 2B) is also shown by the broken line in panel E. In panels B, C, E, and F, cells were treated with FA and azaC in M9 medium. 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.

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 (*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 *priB*, *priC*, and *rep* mutants were the same as those used for other sets of mutants (Fig. 2 to 5). However, azaC concentrations used for the *priA* mutant were much lower than those for other sets of mutants due to an extreme sensitivity of the mutant. The *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 *priA* mutant grew very slowly (see Table S1 in the supplemental material) and was more sensitive to azaC than the *recA* mutant (Fig. 1E). Taken together, it is likely that the extreme azaC sensitivity of the *priA* mutant exhibiting complex phenotypes is attributable to defects not only in RR but also in yet-unknown factors.

Overproduction of Dcm increases azaC sensitivity of cells.

We have previously proposed that DPCs containing large cross-linked proteins (>12 to 14 kDa) are not repaired by NER and exclusively processed by RecBCD-dependent HR that involves the genes *recA*, *recBCD*, *recG*, and *ruvABC*. We confirmed that these mutants were sensitive to FA, whereas the *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 *dcm*-carrying plasmid increased the azaC sensitivity of wt cells by 930-fold and increased that of the *recA* mutant by fivefold (5). However,

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.

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

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.

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.

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*) to azaC without a plasmid (Fig. 2A, F, and D). Similarly, overproduction of Dcm slightly increased the azaC sensitivity of *recA*, *recB*, and *ruvA* mutants that were hypersensitive to azaC without a plasmid (Fig. 2B, C, and E). We infer that the HR of *recA*, *recB*, and *ruvA* 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

FIG. 5. Survival of BER and topoisomerase mutants after treatment with FA and azaC. Cells were treated with indicated agents. (A and B) DNA glycosylase mutants. AB1157 derivatives are as follows: *wt* (filled circles), *fpg* (diamonds), and *nei nth* (squares). KL16 derivatives are as follows: *wt* (filled squares) and *nei nth fpg* (triangles). For a comparison, the survival curve of *recA* (data taken from that shown in Fig. 2B) is also shown by the broken line in panel B. (C and D) AP endonuclease mutants. Shown are data for AB1157 (*wt*) (filled circles) and the *xth nfo* (squares) mutant. (E and F) Topoisomerase mutants. Shown are data for RFM443 (*wt*) (filled circles) and the *gyrB* (squares), and *gyrB topA* (triangles) mutants. Data points showing statistically significant differences ($P < 0.05$) between the *wt* and the mutant are indicated with asterisks.

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, the FA and azaC sensitivities of TLS polymerase mutants (*polB*, *dinB*, and *umuDC*) were assayed. The single mutants exhibited no sensitivity to FA or azaC (Fig. 3A and C). Moreover, the double (*dinB umuDC*) and triple (*polB dinB umuDC*) mutants were not sensitive to FA and azaC at all (Fig. 3B and D). These results clearly indicate that neither direct TLS nor indirect TLS involving template switching (23, 25) provides an alternative damage tolerance pathway 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 KMBL1001 (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. 4J). 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 (*fpg*) account for the major DNA glycosylase activity for oxidized or fragmented pyrimidines (*nth* and *nei*) and purines (*fpg*) (77). Exonuclease III (*xth*) 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 fpg* triple mutant and the *xth nfo* double mutant exhibited a slight but significant FA sensitivity (Fig. 5A and C). The *fpg* 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 fpg* triple mutant and the *xth nfo* double mutant indicate that BER plays at most a minor role in cell survival following FA treatment. Surprisingly, the *nei nth fpg* 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 *fpg* single and *nei nth* double mutants were virtually insensitive to azaC. Also, the *xth 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).

***topA* 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 gyrase subunit B. The mutations confer coumermycin resistance (*Cou^r*) (*gyrB221*) and cause temperature sensitivity (*gyrB203*) (17, 45). The gyrase comprising GyrA (wt) and mutant GyrB (*gyrB203*) has an activity less than 1% of the normal enzyme at 42°C (45). Thus, the *gyrB203* mutation causes partial inactivation of gyrase at 37°C, which was substantiated by the slow-growing phenotype of RFM445 relative to the wt at 37°C (see Fig. S1A in the supplemental material). RFM475 contains the deletion of the *topA* gene encoding Topo I, together with the mutations *gyrB221* and *gyrB203*. 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 gyrase 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 FA nor azaC (Fig. 5E and F). 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

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 sub-pathway 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 monoubiquitinates 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 impli-

cated 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*(Ts) 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*(Ts) 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) *topA* 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 recognize 5-azacytosine (base moiety of azaC) as damage and excise it from DNA. If true, 5-azacytosine left unrepaired in the triple mutant will covalently trap Dcm and thereby increase the azaC sensitivity of the cell. Second, Nei/Nth and Fpg are involved in the repair of the degradation products of 5-azacytosine, since 5-azacytosine is fairly unstable and slowly hydrolyzes to ring fragmentation products (42). Like other ring fragmentation products of DNA bases (32, 33), those of 5-azacytosine left unrepaired 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-azacytosine, degradation products, and Dcm-DPCs in vivo, together with those of the activity of Nei, Nth, and Fpg to 5-azacytosine 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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