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A Weak Adaptive Response to Alkylation Damage in *Salmonella typhimurium*

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An efficient adaptive response to alkylation damage was observed in several enterobacterial species, including *Klebsiella aerogenes*, *Shigella sonnei*, *Shigella boydii*, *Escherichia alkalescens*, *Escherichia hermanii*, and *Escherichia fergusonii*. Increased O^6-methylguanine-DNA and methylphosphotriester-DNA methyltransferase activities correlated with the induction of a 39-kDa protein recognized by monoclonal antibodies raised against the *Escherichia coli* Ada protein. Induced methyltransferase activities were similarly observed in *Aerobacter aerogenes* and *Citrobacter intermedius*, although no antigenically cross-reacting material was present. Weak induction of a 39-kDa protein immunologically related to the *E. coli* Ada protein occurred in *Salmonella typhimurium*. This protein encoded by the cloned *S. typhimurium ada* gene was shown to be an active methyltransferase which repaired O^6-methylguanine and methylphosphotriesters in DNA as efficiently as did the *E. coli* Ada protein. However, the methyltransferase activity of the weakly induced 39-kDa protein in *S. typhimurium* was not detected, apparently because it was self-methylated and thus inactivated during the adaptive *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine pretreatment. In contrast, the *E. coli ada* gene on a low-copy-number plasmid was efficiently induced in *S. typhimurium*, and high methyltransferase activities were observed. We conclude that the inefficient induction of the adaptive response in *S. typhimurium* results from weak transcriptional activation of its *ada* gene by the self-methylated protein.

Monofunctional alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitrosourea (MNU) methylate the bases and phosphate backbone of cellular DNA at several sites. The most harmful base lesions are O^6-methylguanine (O^6-meG), which is a miscoding derivative, and 3-methyladenine, which blocks DNA replication. When *Escherichia coli* is exposed to these methylating agents, an adaptive response is induced which results in the increased expression of four genes, *ada*, *alkB*, *alkA*, and *aidB* (for reviews, see references 15 and 33). A 31-kDa 3-methyladenine-DNA glycosylase II is encoded by the *alkA* gene. The *ada* gene encodes a 39-kDa O^6-meG-DNA methyltransferase I which demethylates O^6-meG, O^4-methylthymine, and the *S*-diastereoisomer of methylphosphotriesters (mePTE) in DNA. The Ada protein transfers the methyl adducts from these lesions onto two of its own cysteine residues. The Ada protein also regulates the adaptive response. Self-methylation at cysteine 69 by transfer of a methyl group from a mePTE in DNA converts the Ada protein into a strong transcriptional activator of the four inducible genes (34, 36). The increased DNA repair capacity which results from induction of the adaptive response enhances the cellular resistance to mutagenesis and killing by methylating agents.

E. coli also has a low level of a constitutive O^6-meG-DNA methyltransferase II, a 19-kDa protein encoded by the *ogt* gene (for a review, see reference 18). In cells not induced for the adaptive response, this enzyme accounts for about 94% of the O^6-meG-DNA methyltransferase activity (23, 26, 37). Ogt is inactivated by self-methylation on repair of DNA damage and thus has the capacity to counteract only low levels of cellular alkylation damage. When cells are exposed to high doses of exogenous alkylating agents, the Ada protein is induced and becomes the major cellular O^6-meG-DNA methyltransferase activity (23, 26, 37).

An adaptive response to alkylation damage has been found in many bacterial species (reviewed in references 5 and 37). In most of these investigations, the response was detected by the increased resistance of cells pretreated with a low dose of methylating agent to the mutagenicity and cell killing of higher doses. The responses of *Bacillus subtilis* and *Micrococcus luteus* have been studied in more detail, and inducible O^6-meG-DNA methyltransferase and 3-methyladenine-DNA glycosylase activities have been characterized (21, 27). Some bacteria, such as *Haemophilus influenzae*, do not have a detectable adaptive response (13). *Salmonella typhimurium* pretreated with a low dose of alkylating agent is sensitized to the mutagenic effects of subsequent challenges (7), which suggests that this bacterium also lacks an adaptive response. This is of particular note because *S. typhimurium* is used for mutagen screening in the Ames test and also because it is closely related to *E. coli*, which has a well-documented response. *S. typhimurium* does, however, contain genomic DNA sequences which hybridize to the *E. coli ada* gene (31). *S. typhimurium* has a low constitutive O^6-meG-DNA repair activity, possibly equivalent to the Ogt protein of *E. coli* (8, 26).

In this work, using immunological and biochemical approaches, we have demonstrated an adaptive response to alkylation damage resulting in enhanced O^6-meG-DNA and mePTE-DNA methyltransferase activities in several previously uninvestigated species of enterobacteria. A weakly induced 39-kDa protein which specifically interacts with anti-Ada monoclonal antibodies was also observed in *S. typhimurium*. However, inducible methyltransferase activity was detected only when the cloned *S. typhimurium ada* gene was present on a multicopy plasmid.

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MATERIALS AND METHODS

Chemicals. MNNG was obtained from Sigma. [^{3}H]MNU (28 Ci/mmol) and ^{125}I-labeled sheep anti-mouse immunoglobulin (750 to 3,000 Ci/mmol) were obtained from Amersham International.

Bacterial strains and plasmids. *E. coli* B/r F26 (*his thy sulA*), *E. coli* K-12 GW7101 (Δ*ada-25*) (32), and *S. typhimurium* LB5010 (r^- m^+) (4), TA1535, and TA2659 were laboratory stocks. Natural isolates of *Escherichia alkalescens* NCTC1601, *E. hermanii* CL157/82, *E. fergusonii* CL796/77, *Klebsiella aerogenes* NCTC418, *Citrobacter intermedius*, *Aerobacter aerogenes*, *Shigella sonnei* NCTC10352, *Shigella boydii* NCTC9355, and *S. typhimurium* NCTC73, NCTC74, NCTC5710, NCTC8298, NCTC8392, and NCTC 10413 were obtained from S. G. Sedgwick. Plasmid pSHR1 contained the *E. coli ada* gene inserted in the vector pHSG415 (10). Plasmids pYG7001 and PYG7034 containing the *S. typhimurium ada* gene were derivatives of pBR322 and pBluescript KS+, respectively (8a), and were obtained from Takehiko Nohmi.

Culture conditions. Cells were grown as specified in Luria broth or M9 minimal medium (20) supplemented as required with thymine (20 μg/ml), histidine (20 μg/ml), and biotin (0.2 μg/ml) at 37°C. Strains carrying low-copy-number pHSG415-derived plasmids were grown at 30°C in the presence of ampicillin at 20 μg/ml. Strains carrying high-copy-number plasmids were grown at 37°C in the presence of ampicillin at 50 μg/ml.

Immunoassay of induction of the Ada protein. Cells were grown in L broth to an A_{600} of 0.2. MNNG was added to culture aliquots to the specified concentration, and the incubation continued for 30 min. One-milliliter cultures were centrifuged and resuspended in 25 μl of detergent buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 2 mM EDTA, 10% glycerol, 5% β-mercaptoethanol, 100 μg of bromophenol blue per ml). Immunoblots were carried out as previously described (37). Briefly, the cellular proteins were resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically to nitrocellulose. The nitrocellulose filter was blocked with 5% dried milk in phosphate-buffered saline and then incubated with two anti-Ada monoclonal antibodies, Mc-A1 and Mc-A2, overnight at 4°C. The filter was subsequently incubated with ^{125}I-labeled sheep anti-mouse immunoglobulins for 5 h at room temperature. The filter was dried and exposed to X-Omat AR or Fuji RX film for 3 to 4 days at room temperature or overnight at −80°C, using intensifying screens and preflashed film.

Enzyme assays. Bacteria were harvested from 50-ml mid-log-phase cultures (A_{600} of 0.5) and disrupted by sonication in 400 μl of 70 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.8)–10 mM dithiothreitol (DTT)–1 mM EDTA–5% glycerol. The O^6-meG-DNA methyltransferase activities of these extracts were assayed by monitoring the demethylation of [^{3}H]O^6-meG residues in [^{3}H]MNU-treated DNA (28 Ci/mmol) (12). Extract (2 to 10 μg) was incubated with this DNA substrate (1,500 cpm) in 100 μl of 70 mM HEPES-KOH (pH 7.8)–10 mM DTT–1 mM EDTA at 37°C for 10 min. Then 20 μg of carrier DNA and 100 μl of 10% trichloroacetic acid were added, and the mixture was kept on ice for 10 min. After centrifugation at 4°C, 200 μl of 0.1 N HCl was added to the pellet, which was then incubated at 70°C for 30 min. The sample was centrifuged, and the radioactivity in the supernatant was determined. MePTE-DNA methyltransferase activity was measured by monitoring the transfer of radioactivity from [^{3}H]MNU-treated poly(dT)-poly(dA) to a protease-sensitive form (19). Using substrate containing 5,000 cpm, the assay procedure was as described above as far as the first precipitation step. Then 200 μl of 0.1 M Tris-HCl (pH 8.0) was added to the pellet along with 200 μg of proteinase K, and the pellet was incubated at 37°C for 2 h. The substrate was precipitated (without adding additional carrier DNA) and centrifuged at 4°C. The radioactivity in the solubilized protein fraction was determined. For monitoring the kinetics of methyl transfer, the reactions were stopped by immersion in a ethanol-dry ice bath before addition of trichloroacetic acid and carrier DNA as described by Lindahl et al. (14).

After incubation of the cell extracts with the [^{3}H]MNU-treated DNA substrate, the ^{3}H-labeled self-methylated Ada and Ogt proteins were resolved by 12% SDS-PAGE and visualized by fluorography (37).

Competent cells and transformation. The techniques were carried out as described by Maniatis et al. (17).

Partial purification of *S. typhimurium* Ada protein. *E. coli* GW7101 (Δ*ada-25*) harboring pYG7034 was grown at 37°C to an A_{600} of 0.85 in 2 liters of L broth. Cells were harvested by centrifugation, washed, and resuspended in 25 ml of buffer A (50 mM Tris-HCl [pH 7.9], 2 mM DTT, 3 mM EDTA, 5% glycerol) containing 0.3 M NaCl. The protein purification procedure was as described for the *E. coli* Ada protein (19). Briefly, the cells were disrupted by ultrasonic treatment, and the cell debris was removed by centrifugation. All procedures were carried out at 4°C. DNA was precipitated from the extract by the addition of neutralized Polymin-P to a final concentration of 0.5%. After centrifugation, ammonium sulfate was added to the supernatant to a final concentration of 55% saturation. The precipitate was collected by centrifugation, resuspended in 1.5 ml of buffer A containing 0.5 M NaCl, and loaded directly onto a column of Ultrogel AcA54. The fractions were monitored by immunoblotting with anti-Ada monoclonal antibodies. A major peak of cross-reacting protein and also of O^6-meG-DNA methyltransferase activity corresponded to an apparent molecular mass of 39 kDa. The most active fractions were pooled, dialyzed overnight against buffer B (30 mM potassium phosphate [pH 7.9], 2 mM DTT, 3 mM EDTA, 50% glycerol) containing 0.3 M NaCl and stored at −80°C. This procedure resulted in a 50-fold purification of the methyltransferase activity.

RESULTS

Frequent occurrence of an adaptive response to alkylation damage in enterobacteria. An initial test for an adaptive response to alkylation damage in a range of enterobacteria was to screen for an inducible protein immunologically related to the Ada protein of *E. coli*. Two monoclonal antibodies which specifically interact with Ada were used (37). After incubation in the presence of MNNG at 1 or 5 μg/ml for 30 min, *K. aerogenes*, *Shigella sonnei*, *Shigella boydii*, *E. alkalescens*, *E. hermanii*, and *E. fergusonii* all displayed an inducible 39-kDa protein which interacted with the anti-Ada monoclonal antibodies (Fig. 1). In *K. aerogenes*, *S. boydii*, and *E. fergusonii*, the 39-kDa protein was detected before induction as a very faint band after a longer exposure of the autoradiograph (data not shown). In these cases, the degree of induction on exposure of the cells to 1 μg of MNNG per ml was at least 10- to 15-fold. Enzyme assays complemented these immunological results and demonstrated that all of these species have inducible O^6-meG-DNA and mePTE-DNA methyltransferase activities (Table

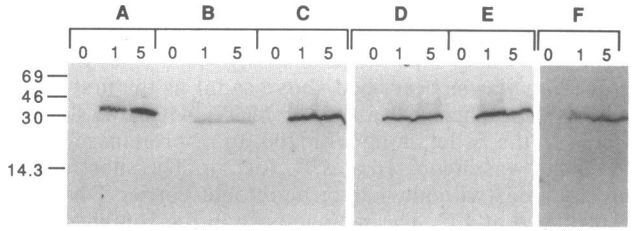

FIG. 1. Recognition of an MNNG-inducible protein by anti-Ada monoclonal antibodies in several enterobacterial species. One-milliliter cultures of *Shigella boydii* (A), *E. hermanii* (B), *E. fergusonii* (C), *E. alkalescens* (D), *Shigella sonnei* (E), and *K. aerogenes* (F) were incubated for 30 min with 0, 1, or 5 μg of MNNG per ml as indicated above each lane. Cell lysates were analyzed by SDS-PAGE and immunoblotted with the anti-Ada monoclonal antibodies Mc-A1 and Mc-A2 and with the secondary antibody ^{125}I-labeled sheep anti-mouse immunoglobulin G. The X-ray film was exposed to the immunoblots for 3 days at room temperature.

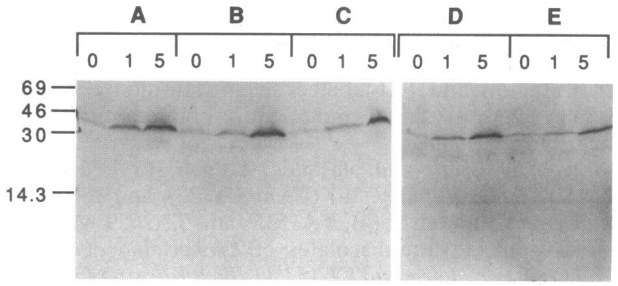

FIG. 2. Recognition of an MNNG-inducible protein in *S. typhimurium* by anti-Ada monoclonal antibodies. One-milliliter cultures of *S. typhimurium* NCTC73 (A), NCTC74 (B), NCTC5710 (C), TA1535 (D), and TA2659 (E) were treated and analyzed as described for Fig. 1. The autoradiographs were exposed for 12 days at room temperature.

1). Although the monoclonal antibodies did not cross-react detectably with any protein in cell lysates of *A. aerogenes* and only faintly detected, after long exposures, a 39-kDa protein in *C. intermedius* (data not shown), inducible O^6-meG-DNA and mePTE-DNA methyltransferase activities were detected and demonstrate an adaptive response to alkylation damage in these bacteria also (Table 1). The estimates of induced mePTE-DNA methyltransferase activity were consistently less than those of O^6-meG-DNA methyltransferase activity. The ratio varied from the expected 1:1 to 1:2 as has been observed previously (19) and may result from instability of the N-terminal domain of the protein (30).

TABLE 1. Enzyme activities

Bacterium	MNNG treatment (1 μg/ml, 30 min)	Activity (U/mg of protein)	
		O^6-meG-methyltransferase	mePTE-methyltransferase
Escherichia coli	−	0.9	ND[a]
	+	11	11
Escherichia alkalescens	−	1.01	ND
	+	14.7	4.2
Escherichia hermanii	−	0.9	ND
	+	7.9	4.3
Escherichia fergusonii	−	2.2	ND
	+	14.8	7.5
Klebsiella aerogenes	−	1.3	ND
	+	13.1	7.6
Shigella sonnei	−	1.3	ND
	+	11.7	7.5
Shigella boydii	−	1.0	ND
	+	14.4	6.9
Citrobacter intermedius	−	0.5	ND
	+	7.9	3.2
Aerobacter aerogenes	−	0.7	ND
	+	9.5	6.5
Salmonella typhimurium	−	0.4	ND
	+	ND	ND
S. typhimurium/pSHR1	−	0.8	0.5
	+	40	27
E. coli GW7101/pSHR1	−	1.0	ND
	+	45	33
E. coli GW7101/pYG7034	−	7.4	4.8
E. coli GW7101/pYG7001	−	1.0	ND
	+	2.7	0.7

[a] ND, not detectable.

A low constitutive O^6-meG-DNA methyltransferase activity but no mePTE-DNA repair activity was observed in all bacteria investigated. These results parallel those obtained in *E. coli* (24, 26, 32), and the constitutive O^6-meG-DNA repair activities are probably equivalent to the Ogt protein of *E. coli*.

A weak adaptive response in *S. typhimurium*. The absence of an adaptive response to alkylation damage in a commonly used laboratory strain of *S. typhimurium* TA1535 was suggested by the data of Guttenplan (7). To determine whether the response was absent from other strains of *S. typhimurium*, six natural isolates, TA2659, and TA1535 were examined for an inducible protein which cross-reacted with the anti-Ada monoclonal antibodies. A 39-kDa protein was detected in all isolates, including the laboratory strains, and was weakly induced on pretreatment of the cells with MNNG. The data for three natural isolates and for TA2659 and TA1535 are shown in Fig. 2. The degree of induction of the 39-kDa protein was 10- to 20-fold less in *S. typhimurium* than in the enterobacterial species analyzed above, a 4-fold-greater exposure time of the autoradiogram being required to clearly visualize the induced protein in *S. typhimurium*. The 39-kDa protein was fully induced in the enterobacteria by MNNG at 1 μg/ml, whereas *S. typhimurium* required at least 5 μg/ml for the maximum but weak induction.

Absence of detectable inducible DNA-methyltransferase activities in *S. typhimurium*. O^6-meG-DNA methyltransferase assays revealed a constitutive activity in *S. typhimurium* TA1535, in agreement with previous observations (26) and comparable to that observed in the other enterobacteria (Table 1). However, no activity was detected in extracts of cells pretreated with MNNG at 1 μg/ml (Table 1). MePTE-DNA repair activity was not detected in extracts of either untreated or MNNG-pretreated cells (Table 1). The presence of the weakly induced 39-kDa protein therefore did not correlate with demonstrable elevated O^6-meG-DNA and mePTE-DNA methyltransferase activities. Self-methylation of DNA methyltransferases on repair of O^6-meG and mePTE in DNA results in their inactivation. The apparent absence of activity in the pretreated cells thus signified inactivation of the constitutive enzyme and also the weakly induced 39-kDa protein as a result of the MNNG pretreatment. In an attempt to induce the response without total inactivation of these proteins, several pretreatment doses were used. As the pretreatment dose of MNNG was de-

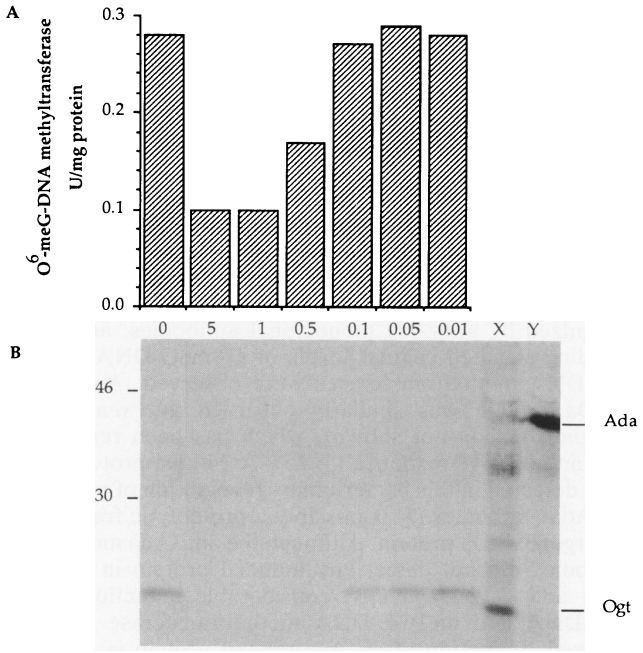

FIG. 3. Absence of enhanced O^6-meG-DNA methyltransferase activity in MNNG-pretreated *S. typhimurium*. (A) *S. typhimurium* TA1535 was grown in minimal medium and treated for 1 h with the doses of MNNG (micrograms per milliliter) indicated below the histogram. O^6-meG-DNA methyltransferase activity was assayed in crude cell extracts. (B) The ^{3}H-labeled self-methylated methyltransferases arising during these assays were visualized by SDS-PAGE and fluorography. *S. typhimurium* TA1535 was pretreated with the doses of MNNG indicated above the lanes. *E. coli* F26 was untreated (lane X) or pretreated with 1 μg of MNNG per ml (lane Y).

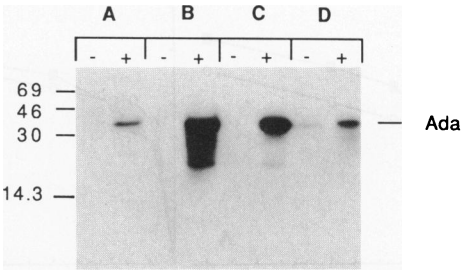

FIG. 4. Induction of expression of the cloned *S. typhimurium ada* gene. Cultures of *S. typhimurium* LB5010 (A), *S. typhimurium* LB5010/pSHR1 (B), *E. coli* GW7101/pSHR1 (C), and *E. coli* GW7101/pYG7001 (D) were untreated (−) or pretreated with MNNG (1 μg/ml) (+), and cell extracts (100 μg of protein) were analyzed by SDS-PAGE and immunoblotting as described for Fig. 1. The X-ray film was exposed to the immunoblot by using intensifying screens overnight at −80°C.

creased from 5 to 0.05 μg/ml, the measured methyltransferase activity returned to the level characteristic of untreated cells (Fig. 3A). High cell extract concentrations were used in these assays. The lowest activity observed, 0.1 U of O^6-meG-DNA methyltransferase per mg of protein, possibly resulted from nonspecific DNA degradation and was thus the limit of detection of the assays. An elevated activity above the constitutive level as a result of the induced 39-kDa protein was not observed at any of the pretreatment doses used. At low pretreatment doses of MNNG such as 0.1 μg/ml, the total detectable methyltransferase activity could be composed of both the constitutive enzyme and the induced 39-kDa protein which has been only partially inactivated by the pretreatment dose. To investigate this possibility, the extracts after incubation with ^{3}H-labeled methylated DNA were resolved on an SDS-polyacrylamide gel. The ^{3}H-labeled self-methylated proteins were visualized by fluorography (Fig. 3B). As the MNNG pretreatment dose was decreased, a labeled protein of approximately 20 kDa appeared and returned to the level in untreated cells. However, no labeled protein of 39 kDa was observed. These observations suggest that pretreatment of *S. typhimurium* with MNNG at 1 or 5 μg/ml induces the 39-kDa protein sufficiently to be detected by the immunoassay but that it is inactivated by the pretreatment regimen and therefore cannot be detected as a ^{3}H-labeled methylated protein. At lower MNNG doses, the protein is not induced or is so weakly induced that it again cannot be detected as a ^{3}H-labeled self-methylated protein. Induction of the Ada protein in *E. coli* was clearly observed by this approach on pretreatment of cells with MNNG at 1 μg/ml (Fig. 3B, lane Y). The Ogt protein was not detected in these induced cells because it was inactivated a result of the MNNG pretreatment (Fig. 3B, lane Y). The constitutive methyltransferase of *S. typhimurium* was slightly larger on SDS-PAGE than the 19-kDa Ogt protein of *E. coli* observed in untreated cells (Fig. 3B, lane X).

Step-up adaptation, whereby cells are exposed to a series of low but increasing doses of MNNG, has been used previously to induce the adaptive response in *M. luteus* and in various mutant strains of *E. coli* K-12 which are sensitive to alkylation toxicity (2, 11). This means of adapting *S. typhimurium* by using MNNG at 0.1 μg/ml for 60 min and then at 0.5 μg/ml for 60 min also failed to yield detectable elevated levels of either O^6-meG or mePTE repair activity corresponding to the increase in the 39-kDa protein observed immunologically (data not shown).

To demonstrate that the lack of an induced methyltransferase activity was not a result of inhibitory factors in extracts of MNNG-pretreated *S. typhimurium*, purified *E. coli* Ada protein (0.05 U) was assayed for mePTE-DNA methyltransferase activity in the presence of 12.5 μl of *S. typhimurium* extract (4 mg of protein per ml). The Ada protein was fully active and demonstrated the absence of methyltransferase inhibitors in the cell extracts (data not shown).

Induction of *E. coli ada* gene expression in *S. typhimurium*. A plasmid carrying the *E. coli ada* gene was transformed into *S. typhimurium* to investigate whether expression of this gene was fully inducible and its product assayable in *S. typhimurium*. Strain LB5010 (r^- m^+) was transformed with pSHR1, which is a low-copy-number plasmid and carries the *E. coli ada* gene. A low constitutive level of O^6-meG and mePTE-DNA methyltransferase activities was found in cell extracts of LB5010/pSHR1. On pretreatment of the cells with MNNG, these activities increased approximately 50-fold. The extent of induction of the *E. coli ada* gene was comparable with that observed in *E. coli* GW7101 (Δ*ada-25*)/pSHR1 (Table 1). Immunoassays performed in parallel also showed high levels of induced Ada protein in both *E. coli* and *S. typhimurium* (Fig. 4). Expression of the *E. coli ada* gene from this plasmid and detection of its product were therefore not inhibited in *S. typhimurium*.

Detection of methyltransferase activity on expression of the

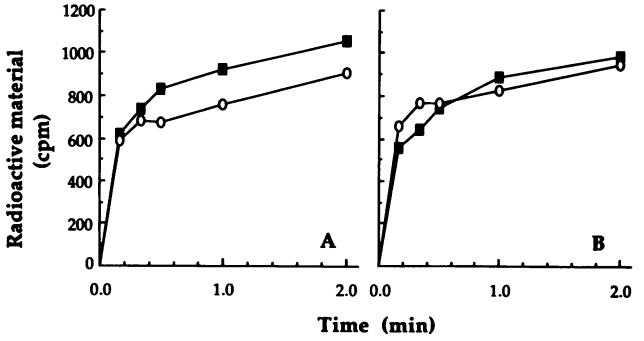

FIG. 5. Reaction kinetics of DNA repair by the *S. typhimurium* and *E. coli* Ada proteins. The methyltransferase activities of the purified *E. coli* Ada protein (○) and the partially purified *S. typhimurium* Ada protein (■) were monitored at 37°C by incubation with [^{3}H]MNU-treated substrates. Samples were taken at various times, and the reactions were stopped immediately by immersion in an ethanol-dry ice bath. (A) O^6-meG-DNA methyltransferase activity assayed by the removal of radioactive material from [^{3}H]MNU-treated DNA; (B) mePTE-DNA methyltransferase activity assayed by the transfer of radioactive material from [^{3}H]MNU-treated poly(dA)-poly(dT) to a protease-sensitive form.

***Salmonella ada* gene from a multicopy plasmid.** Plasmid pYG7034 contains the *S. typhimurium ada* gene under control of the *lac* promoter inserted into the high-copy-number vector pBluescript KS+ (8a). Both O^6-meG-DNA and mePTE-DNA methyltransferase activities were observed in cell extracts of *E. coli* GW7101 (*Δada-25*)/pYG7034 without pretreatment with isopropyl-β-D-thiogalactopyranoside (IPTG). Thus, only by expression of the *S. typhimurium ada* gene from a high-copy-number plasmid was it possible to demonstrate that the product of this gene was active in repairing O^6-meG and mePTE in DNA (Table 1). Plasmid pYG7001 is a pBR322 derivative which contains the *S. typhimurium ada* gene under control of its own promoter (8a). A low constitutive level of O^6-meG-DNA repair activity was observed in extracts of GW7101/pYG7001. On pretreatment of the cells with MNNG, the O^6-meG repair activity increased two- to threefold, and a low level of mePTE-DNA methyltransferase activity was also observed (Table 1). An increase in these activities resulting from induced expression of the *S. typhimurium ada* gene was therefore detected. However, the activities were very low. An immunoblot displayed a 10-fold increase in the amount of cellular Ada protein (Fig. 4D). Similar plasmid derivatives carrying the *E. coli ada* gene result in much higher cellular levels of the methyltransferase activity both before and after induction (29, 35).

Rapid DNA repair by the *S. typhimurium* Ada protein. A possible explanation for the weak inducibility of the *S. typhimurium ada* gene was that the *S. typhimurium* Ada protein was slow in demethylating alkylated DNA and was therefore converted inefficiently into a positive regulator. To examine the kinetics of DNA repair by the *S. typhimurium* Ada protein, it was first necessary to separate this protein from the constitutive methyltransferase activity. The *S. typhimurium* Ada protein was therefore partially purified by ammonium sulfate precipitation and gel filtration on a AcA54 column, using *E. coli* GW7101 (*Δada-25*)/pYG7034 as the source of enzyme. The rates of repair of O^6-meG and mePTE in DNA by the purified *E. coli* Ada protein and the partially purified *S. typhimurium* Ada protein were compared (Fig. 5). The two proteins repaired both types of adducts at comparable rates. The *S. typhimurium* Ada protein was therefore as efficient as the *E. coli* Ada protein in repairing these DNA lesions.

DISCUSSION

An adaptive response to alkylation damage has been demonstrated in several enterobacterial species, including *K. aerogenes*, *Shigella sonnei*, *Shigella boydii*, *E. alkalescens*, *E. hermanii*, and *E. fergusonii*. An inducible protein of 39 kDa, the same size as the Ada protein of *E. coli*, was recognized by anti-Ada monoclonal antibodies, and corresponding elevated cellular levels of O^6-meG-DNA and mePTE-DNA methyltransferases were observed. An induced 39-kDa protein was similarly observed in *Pseudomonas aeruginosa* (data not shown), which has been reported to have an adaptive response (5, 25). A 24-kDa protein previously detected in this bacterium by recognition of polyclonal anti-Ada antibodies (5) is possibly a proteolytic fragment of the larger 39-kDa protein. Although the anti-Ada monoclonal antibodies did not detect any induced protein in *A. aerogenes* and *C. intermedius*, considerable induction of O^6-meG-DNA and mePTE-DNA methyltransferase activities occurred. Induction of an Ada-related protein is therefore apparent in all of these bacterial species. The occurrence of genomic DNA sequences which hybridize to the *E. coli ada* gene had been reported in *K. aerogenes*, *Shigella sonnei*, *E. alkalescens*, and *C. intermedius* (31).

In *E. coli*, induction of the Ada methyltransferase results in cellular resistance to the mutagenicity of methylating agents. Induction of this protein in the other enterobacterial species indicates that they will also acquire this enhanced resistance. In *E. coli*, the Ada protein positively regulates expression of three other genes. It is unknown whether the enterobacteria have inducible homologs of the *alkA*, *alkB*, and *aidB* genes.

A 39-kDa protein was induced, although weakly, in *S. typhimurium*. In this case, increased methyltransferase activities could not be detected, apparently because the 39-kDa protein was inactivated by self-methylation on repair of DNA damage resulting from the inducing MNNG pretreatment. When the MNNG inducing dose was decreased, the level of induction of the 39-kDa protein also decreased. The rate of synthesis of new active protein was therefore never quite sufficient for it to accumulate in the cell before it was rapidly consumed in DNA repair. The absence of accumulated active methyltransferase agrees with a previous report which failed to detect induced cellular resistance to the mutagenicity of MNNG in *S. typhimurium* (7).

The *S. typhimurium ada* gene has been isolated recently by T. Nohmi (8a). A means of achieving high cellular levels of the 39-kDa protein without pretreatment of the cells with MNNG was therefore available by overexpression of the cloned gene. In this case, high cellular O^6-meG-DNA and mePTE-DNA methyltransferase activities were detected. The product of the *S. typhimurium ada* gene is therefore an active DNA repair enzyme. MNNG induction of the cloned gene inserted in pBR322 resulted in a small but measurable increase in the cellular methyltransferase activities. The plasmid-encoded Ada protein would also be consumed to the same extent by the adaptive treatment but must have been present in sufficient amounts after induction of multiple gene copies that residual active protein could be detected.

The Ada protein of *E. coli* is converted into a strong transcriptional activator by self-methylation on repair of

mePTEs in DNA. If a similar regulatory mechanism exists in *S. typhimurium*, weak induction could result from inefficient repair of mePTEs by the Ada protein or poor gene activation by the self-methylated protein. The partially purified *S. typhimurium* Ada protein was shown to be as efficient in repairing mePTEs in DNA as the *E. coli* Ada protein. It is thus proposed that the weak induction of the adaptive response in *S. typhimurium* results from poor transcriptional activation by the self-methylated Ada protein.

In addition to the inducible methyltransferase, a constitutive O^6-meG-DNA methyltransferase was present in all of the enterobacterial species examined. This activity may protect cells against endogenous alkylating agents, such as *S*-adenosylmethionine (3, 28), and low levels of exogenous agents.

The cross-reaction of the monoclonal antibodies with Ada-methyltransferases of various enterobacterial species signifies conservation of the antigenic epitope(s). The two monoclonal antibodies were used separately with *S. typhimurium* extracts, and both recognized the 39-kDa protein. The epitope recognized by Mc-A1 has been located between amino acids 120 and 149 in the amino-terminal domain of the *E. coli* Ada protein, and that recognized by Mc-A2 is located between amino acids 315 and 354 at the carboxy terminus (37). The antibodies did not interact with the AdaA and AdaB proteins of *B. subtilis* (data not shown), the Ogt protein of *E. coli*, or the constitutive methyltransferases of the other enterobacterial species. AdaA and AdaB of *B. subtilis* have equivalent functions and show high amino acid identity to the amino- and carboxy-terminal domains of Ada, respectively (21). Ada, AdaB, and Ogt, which all repair O^6-meG, are highly conserved around their active cysteine residue (21). Their amino acid sequences are most divergent in the carboxy-terminal amino acids, in the case of Ada, the last nine amino acids. It is therefore most probable that Mc-A2 recognizes an epitope in this region which must have retained some conservation within the enterobacterial species.

An adaptive response to alkylation damage has now been observed in a wide range of bacterial species but has been reported to be absent from several other bacteria and microorganisms (reviewed in references 5 and 37). The detection of a response in *S. typhimurium* by methods other than a test for enhanced resistance to alkylation mutagenicity suggests that weak inducible responses may have been missed in some organisms. An immunological test as described here or a screen for increased transcription may be more sensitive assays for detection of an adaptive response. In this respect, *Saccharomyces cerevisiae* has been reported to lack an adaptive response (16, 22), but inducibility of a gene which encodes a 3-methyladenine-DNA glycosylase has been recently observed by Northern (RNA) blotting (6). This report and that of a response in the fungus *Aspergillus nidulans* (9) indicate that inducible responses to alkylation damage may be present in lower eukaryotes.

The extents of known responses in *B. subtilis*, *E. coli*, *M. luteus*, and *S. typhimurium* differ greatly and may be related to the requirement for the responses in particular environments. It is of note that bacteria with strong adaptive responses can exist as soil organisms, which suggests that exposure to alkylating agents may be greater in this environment. Several environmental methylating agents have been described (37).

S. typhimurium is used widely for mutagen detection by the Ames test (1). The use of *E. coli* or any other bacteria with an effective adaptive response in such tests would result in failure to detect a major class of mutagens, the mutagenic methylating agents. The choice of *S. typhimurium* with a weak adaptive response was therefore fortunate. *E. coli ada* mutants deficient in the response can now also be used in such screening tests.

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REFERENCES

1. **Ames, B. N., J. McCann, and E. Yamasaki.** 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res. **31**:347–364.
2. **Ather, A., Z. Ahmed, and S. Riazuddin.** 1984. Adaptive response of *Micrococcus luteus* to alkylating chemicals. Nucleic Acids Res. **12**:2111–2126.
3. **Barrows, L. R., and P. N. Magee.** 1982. Nonenzymatic methylation of DNA by S-adenosylmethionine *in vitro*. Carcinogenesis **3**:349–351.
4. **Bullas, L. R., and J. Ryu.** 1983. *Salmonella typhimurium* LT2 strains which are r^- m^+ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. **156**:471–474.
5. **Ceccoli, J., N. Rosales, M. Goldstein, and D. B. Yarosh.** 1988. Polyclonal antibodies against O^6-methylguanine-DNA methyltransferase in adapted bacteria. Mutat. Res. **194**:219–226.
6. **Chen, J., B. Derfler, and L. Samson.** 1990. *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase has homology to the AlkA glycosylase of *E. coli* and is induced in response to DNA alkylation damage. EMBO J. **9**:4569–4575.
7. **Guttenplan, J. B.** 1979. Comutagenic effects exerted by N-nitroso compounds. Mutat. Res. **66**:25–32.
8. **Guttenplan, J. B., and S. Milstein.** 1982. Resistance of *Salmonella typhimurium* TA1535 to O^6-guanine methylation and mutagenesis induced by low doses of N-methyl-N′-nitro-N-nitrosoguanidine: an apparent constitutive repair activity. Carcinogenesis **3**:327–331.

8a. **Hakura, A., K. Morimoto, T. Sofuni, and T. Nohmi.** 1991. Cloning and characterization of the *Salmonella typhimurium ada* gene, which encodes O^6-methylguanine-DNA methyltransferase. J. Bacteriol. **173**:3663–3672.

9. **Hooley, P., S. G. Shawcross, and P. Strike.** 1988. An adaptive response to alkylating agents in *Aspergillus nidulans*. Curr. Genet. **14**:445–449.
10. **Hughes, S. J., and B. Sedgwick.** 1989. The adaptive response to alkylation damage: constitutive expression through a mutation in the coding region of the *ada* gene. J. Biol. Chem. **264**:21369–21375.
11. **Jeggo, P.** 1979. Isolation and characterization of *Escherichia coli* K-12 mutants unable to induce the adaptive response to simple alkylating agents. J. Bacteriol. **139**:783–791.
12. **Karran, P., T. Lindahl, and B. Griffin.** 1979. Adaptive response to alkylating agents involves alteration *in situ* of O^6-methylguanine residues in DNA. Nature (London) **280**:76–77.
13. **Kimball, R. F.** 1980. Further studies on the induction of mutation in *Haemophilus influenzae* by N-methyl-N′-nitro-N-nitrosoguanidine: lack of an inducible error-free repair system and the effect of exposure medium. Mutat. Res. **72**:361–372.
14. **Lindahl, T., B. Demple, and P. Robins.** 1982. Suicide inactivation of the *E. coli* O^6-methylguanine-DNA methyltransferase. EMBO J. **1**:1359–1363.
15. **Lindahl, T., B. Sedgwick, M. Sekiguchi, and Y. Nakabeppu.** 1988. Regulation and expression of the adaptive response to alkylating agents. Annu. Rev. Biochem. **57**:133–157.
16. **Maga, J. A., and K. McEntee.** 1985. Response of *S. cerevisiae* to N-methyl-N′-nitro-N-nitrosoguanidine: mutagenesis, survival and DDR gene expression. Mol. Gen. Genet. **200**:313–321.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Margison, G. P., D. P. Cooper, and P. M. Potter.** 1990. The *E. coli ogt* gene. Mutat. Res. **233**:15–21.
19. **McCarthy, T. V., and T. Lindahl.** 1985. Methyl phosphotriesters in alkylated DNA are repaired by the Ada regulatory protein of *E. coli*. Nucleic Acids Res. **13**:2683–2698.
20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Morohoshi, F., K. Hayashi, and N. Munakata.** 1990. *Bacillus subtilis ada* operon encodes two DNA alkyltransferases. Nucleic Acids Res. **18**:5473–5480.
22. **Polakowska, R., G. Perozzi, and L. Prakash.** 1986. Alkylation mutagenesis in *Saccharomyces cerevisiae*: lack of evidence for an adaptive response. Curr. Genet. **10**:647–655.
23. **Potter, P. M., K. Kleibl, L. Cawkwell, and G. P. Margison.** 1989. Expression of the *ogt* gene in wild type and *ada* mutants of *E. coli*. Nucleic Acids Res. **17**:8047–8060.
24. **Potter, P. M., M. C. Wilkinson, J. Fitton, F. J. Carr, J. Brennand, D. P. Cooper, and G. P. Margison.** 1987. Characterization and nucleotide sequence of *ogt*, the O^6-alkylguanine-DNA alkyltransferase gene of *E. coli*. Nucleic Acids Res. **15**:9177–9193.
25. **Rasool, S. A., A. Mirza, and M. A. Khan.** 1990. Nitrosoguanidine-induced adaptive repair in *Pseudomonas aeruginosa*. Curr. Genet. **17**:417–419.
26. **Rebeck, G. W., C. M. Smith, D. L. Goad, and L. Samson.** 1989. Characterization of the major DNA repair methyltransferase activity in unadapted *Escherichia coli* and identification of a similar activity in *Salmonella typhimurium*. J. Bacteriol. **171**: 4563–4568.
27. **Riazuddin, S., A. Athar, and A. Sohail.** 1987. Methyl transferases induced during chemical adaptation of *M. luteus*. Nucleic Acids Res. **15**:9471–9486.
28. **Rydberg, B., and T. Lindahl.** 1982. Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO J. **1**:211–216.
29. **Sedgwick, B.** 1983. Molecular cloning of a gene which regulates the adaptive response to alkylating agents in *Escherichia coli*. Mol. Gen. Genet. **191**:466–472.
30. **Sedgwick, B., P. Robins, N. Totty, and T. Lindahl.** 1988. Functional domains and methyl acceptor sites of the *Escherichia coli* Ada protein. J. Biol. Chem. **263**:4430–4433.
31. **Sedgwick, S. G., and P. A. Goodwin.** 1985. Differences in mutagenic and recombinational DNA repair in enterobacteria. Proc. Natl. Acad. Sci. USA **82**:4172–4176.
32. **Shevell, D. E., Z. A. Abou, B. Demple, and G. C. Walker.** 1988. Construction of an *Escherichia coli* K-12 *ada* deletion by gene replacement in a *recD* strain reveals a second methyltransferase that repairs alkylated DNA. J. Bacteriol. **170**:3294–3296.
33. **Shevell, D. E., B. M. Friedman, and G. C. Walker.** 1990. Resistance to alkylation damage in *Escherichia coli*: role of the Ada protein in induction of the adaptive response. Mutat. Res. **233**:53–72.
34. **Takano, K., Y. Nakabeppu, and M. Sekiguchi.** 1988. Functional sites of the Ada regulatory protein of *Escherichia coli*: analysis by amino acid substitutions. J. Mol. Biol. **201**:261–271.
35. **Teo, I., B. Sedgwick, B. Demple, B. Li, and T. Lindahl.** 1984. Induction of resistance to alkylating agents in *E. coli*: the ada^+ gene product serves both as a regulatory protein and as an enzyme for repair of mutagenic damage. EMBO J. **3**:2151–2157.
36. **Teo, I., B. Sedgwick, M. W. Kilpatrick, T. V. McCarthy, and T. Lindahl.** 1986. The intracellular signal for induction of resistance to alkylating agents in *E. coli*. Cell **45**:315–324.
37. **Vaughan, P., B. Sedgwick, J. Hall, J. Gannon, and T. Lindahl.** 1991. Environmental mutagens which induce the adaptive response to alkylation damage in *Escherichia coli*. Carcinogenesis **12**:263–268.