JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1511–1519 Vol. 177, No. 6
0021-9193/95/$04.00+0

Construction and Characterization of Mutants of *Salmonella typhimurium* Deficient in DNA Repair of O^6-Methylguanine

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Received 7 October 1994/Accepted 4 January 1995

***Escherichia coli* has two O^6-methylguanine DNA methyltransferases that repair alkylation damage in DNA and are encoded by the *ada* and *ogt* genes. The *ada* gene of *E. coli* also regulates the adaptive response to alkylation damage. The closely related species *Salmonella typhimurium* possesses methyltransferase activities but does not exhibit an adaptive response conferring detectable resistance to mutagenic methylating agents. We have previously cloned the *ada*-like gene of *S. typhimurium* (ada_{ST}) and constructed an ada_{ST}-deletion derivative of *S. typhimurium* TA1535. Unexpectedly, the sensitivity of the resulting strain to the mutagenic action of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) was similar to that of the parent strain. In this study, we have cloned and sequenced the *ogt*-like gene of *S. typhimurium* (ogt_{ST}) and characterized ogt_{ST}-deletion derivatives of TA1535. The ogt_{ST} mutant was more sensitive than the parent strain to the mutagenicity of MNNG and other simple alkylating agents with longer alkyl groups (ethyl, propyl, and butyl). The ada_{ST}-ogt_{ST} double mutant had a level of hypersensitivity to these agents similar to that of the ogt_{ST} single mutant. The ogt_{ST} and the ada_{ST}-ogt_{ST} mutants also displayed a two to three times higher spontaneous mutation frequency than the parent strain and the ada_{ST} mutant. These results indicate that the Ogt_{ST} protein, but not the Ada_{ST} protein, plays a major role in protecting *S. typhimurium* from the mutagenic action of endogenous as well as exogenous alkylating agents.**

Of the DNA lesions generated by methylating agents, a minor alkylation product O^6-methylguanine (O^6-MeG) is responsible for most of the mutations induced (22). This altered base directs the incorporation of either thymine or cytosine, without blocking DNA replication, resulting in GC-to-AT transition mutations (3, 21, 50). To counteract such mutagenic effects, most organisms possess O^6-MeG DNA methyltransferases (MTs) that directly transfer the methyl group from O^6-MeG to a cysteine residue within the protein in an autoinactivating stoichiometric fashion (19, 20, 32). The ubiquity of constitutive MTs in species ranging from bacteria to mammals (9, 15, 29, 36, 37, 49, 59) may indicate the occurrence of methylating agents as endogenous as well as exogenous mutagens.

When *Escherichia coli* is exposed to subtoxic doses of methylating or ethylating agents, an adaptive response which results in the increased expression of four genes, *ada*, *alkB*, *alkA*, and *aidB*, is induced (11, 42). The *ada* gene encodes a 39-kDa MT which transfers the methyl group from O^6-MeG and O^4-methylthymine (O^4-MeT) to its Cys-321 residue as well as from one of the stereoisomers of methylphosphotriesters to residue Cys-69 (4, 25–27, 32). Methylation at residue Cys-69 converts the Ada protein into an efficient transcriptional activator of the four inducible genes including the *ada* gene itself (52, 54). The increased repair capacity which results from induction of the adaptive response enhances cellular resistance to the mutagenic and killing effects of methylating agents. Besides the Ada protein, *E. coli* possesses a second MT, the Ogt protein, which accounts for about 95% of the MT activity in cells not induced for the adaptive response (35, 38, 40, 47). The Ogt protein repairs O^6-MeG and O^4-MeT but not methylphosphotriesters and can repair O^6-ethylguanine in DNA at a rate higher than that of the Ada protein (57). The amino acid sequence of this 19-kDa protein shares homology with the C-terminal half of the 39-kDa Ada protein (4, 30, 35). The *ogt* gene is expressed constitutively (34) and is located at 29 min on the *E. coli* genetic map (33, 53), whereas the *ada* gene is located at 47 min (14, 45).

In contrast to the response in *E. coli*, an adaptive response conferring resistance to alkylating agents is not detectable in *Salmonella typhimurium* (6, 7, 20), although weak induction of an Ada protein is observed (55). To clarify the molecular mechanism resulting in the lack of an effective adaptive response in *S. typhimurium*, we previously cloned an *ada*-like gene from *S. typhimurium* (ada_{ST}) and constructed an ada_{ST}-deletion mutant (8, 60). Unlike *E. coli ada* mutants, which are very sensitive to the mutagenic effect of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) (11), the ada_{ST}-deletion derivative exhibited almost the same sensitivity as its wild-type strain (60). These results led us to suggest that a constitutive Ogt_{ST} MT, and not the Ada_{ST} protein, may play a major role in protecting *S. typhimurium* against the mutagenic effects of alkylating agents.

In this study, we have cloned the ogt_{ST} gene and constructed ogt_{ST} and ada_{ST}-ogt_{ST}-deletion derivatives. The ogt_{ST} gene was disrupted by the preligation method that we have developed recently (60). The ogt_{ST}-deletion derivatives exhibited hypersensitivities not only to mutagenic methylating agents but also to mutagenic ethylating, propylating, and butylating agents. *S. typhimurium* TA1535 is used as the tester strain in the Ames mutagenicity test (24). Inclusion of the ogt_{ST}-deletion mutants of TA1535 in these tests would substantially increase the sen-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
S. typhimurium		
LB5000	*metA22 metE551 trpC2 ilv-452* H1-b H2-e,n,x *fla-66 rpsL120 xyl-404 leu hsdL6 hsdSA29 hsdSB*	K. E. Sanderson
TA1535	*hisG46 rfa* Δ*uvrB*	B. N. Ames
YG7100	Same as TA1535 but Δada_{ST}::Km^r	60
YG7104	Same as TA1535 but Δogt_{ST}::Cm^r	This study
YG7108	Same as TA1535 but Δada_{ST}::Km^r Δogt_{ST}::Cm^r	This study
E. coli		
AB1157	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY tsx-33 qsr'* mutant *supE44 galK2*, λ$^-$, *rac hisG4*(Oc) *rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3*(Oc) *thi-1*	G. C. Walker
CSR603	*recA1 uvrA6 phr-1*	G. C. Walker
GW7101	Same as AB1157 but Δ*ada*::Cm^r	G. C. Walker
KT233	Same as AB1157 but Δ*ada*::Km^r Δ*ogt*::Cm^r	53
LE392	*hsdR514 supE44 supF58*, host strain for phage propagation	G. C. Walker
XL-1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}]*ZΔM15* Tn*10*(Tc^r)	Stratagene
Plasmids		
pACYC184	Plasmid carrying Cm^r gene cartridge, Cm^r Tc^r	Laboratory stock
pBluescriptKS+	Vector for subcloning, Ap^r	Stratagene
pBR322	Vector for cloning, Ap^r Tc^r	Laboratory stock
pUC18	Vector for subcloning, Ap^r	Nippon Gene
pUC19	Vector for subcloning, Ap^r	Nippon Gene
pYG601	Same as pBluescriptKS+ but carries *E. coli ogt* on a 2.95-kb *Eco*RI fragment of λ261 phage DNA, Ap^r	This study
pYG603	Same as pBR322, but carries *E. coli ogt* on a 2.95-kb *Eco*RI fragment of λ261 phage DNA, Ap^r Tc^r	This study
pYG607	Same as pBR322 but carries a 5.7-kb *Sau*3AI fragment of TA1538 chromosomal DNA at its *Bam*HI site, Ap^r	This study
pYG609	Same as pBR322 but carries a 5.9-kb *Sau*3AI fragment of TA1538 chromosome DNA at its *Bam*HI site, Ap^r	This study
pYG611	Same as pBR322 but carries a 1.3-kb *Eco*RV fragment of pYG609, Ap^r	This study
pYG612	Same as pYG611 but the fragment is inserted in opposite orientation, Ap^r	This study
pYG613	Same as pBR322 but carries a 3.4-kb *Sau*3AI fragment of TA1538 chromosomal DNA at its *Bam*HI site, Ap^r	This study
pYG616	Same as pBR322 but carries a 4.4-kb fragment derived from pYG609 between its *Eco*RI and *Nru*I sites, Ap^r	This study
pYG617	Same as pUC19 but carries a 1.5-kb *Sph*I-*Sph*I fragment derived from pYG613, Ap^r	This study
pYG618	Same as pUC18 but carries a 1.5-kb *Sph*I-*Sph*I fragment derived from pYG613, Ap^r	This study
pYG620	Same as pYG616 but a Cm^r gene (1.3 kb) replaces the *Eco*RI-*Sph*I fragment (1.3 kb) containing ogt_{ST} and is between flanking regions of ogt_{ST} gene, Ap^r Cm^r	This study
pYG7034	pBluescript KS+ carrying ada_{ST} in its *Eco*RV site, Ap^r	8

sitivity of detection of environmental mutagenic alkylating agents. The roles of the Ogt_{ST} protein in protecting *S. typhimurium* from exogenous and endogenous mutagenic alkylating agents are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. Strains and plasmids used in this study are listed in Table 1. Lambda phages that carry fragments of the chromosome of *E. coli* were obtained from Kohara et al. (16). The lambda clone 3G3 carrying the *E. coli ogt* gene was amplified in strain LE392 of *E. coli*.

Media and chemicals. Luria-Bertani medium was used for routine growth of bacteria and supplemented, when necessary, with ampicillin (50 μg/ml), chloramphenicol (10 μg/ml), or kanamycin (25 μg/ml) for plasmid selection and maintenance. Agar plates containing MNNG were prepared as described previously (60). Histidine-fortified Vogel-Bonner medium was prepared as described by Maron and Ames (24), with the addition of L-histidine (50 μg/ml) and D-biotin (3 nmol/ml). MNNG, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG), *N*-propyl-*N*′-nitro-*N*-nitrosoguanidine (PNNG), and *N*-butyl-*N*′-nitro-*N*-nitrosoguanidine (BNNG) were provided by M. Nakadate (National Institute of Health Sciences, Tokyo, Japan).

Preparation of *ogt* gene of *E. coli*. The *ogt* gene of *E. coli* was prepared according to the method of Takano et al. (53). Briefly, lambda DNA was prepared from a lysate of LE392 carrying the 3G3 phage clone, and the *Eco*RI-*Eco*RI 2.95-kb fragment was purified and subcloned into the *Eco*RI site of the vector pBR322. The resulting plasmid, pYG603, conferred resistance to the cytotoxicity of MNNG to *E. coli* KT233, in which the *ogt* and *ada* genes have been deleted.

Cloning of ogt_{ST}. A gene library of *S. typhimurium* TA1538, previously constructed by Watanabe et al. (56), was modified in the restrictionless XL-1 Blue strain of *E. coli*. *E. coli* KT233 was transformed with this DNA library, spread directly on agar plates containing MNNG (1.5 μg/ml), and incubated for 2 days at 37°C. Plasmid DNA was isolated from each surviving colony and reintroduced into KT233. The resulting transformants were examined for their resistance to the cell-killing effects of MNNG. A physical map of each plasmid that conferred MNNG resistance to strain KT233 was constructed. Clones showing the same restriction pattern as that of the ada_{ST} gene and/or hybridized with an ada_{ST} probe were excluded from the candidates for the ogt_{ST} gene. The remaining 12 clones, all of which hybridized with the *E. coli ogt* gene, were subjected to further study.

DNA sequencing and labeling of plasmid-encoded proteins in maxicells. A 1.6-kb *Sph*I-*Sph*I DNA fragment was purified from plasmid pYG613, one of the 12 candidate clones for the ogt_{ST} gene. This DNA fragment was subcloned into pUC18 and pUC19, resulting in plasmids pYG618 and pYG617, respectively. Sets of deletion derivatives of pYG617 and pYG618 were generated, and both strands of a 0.8-kb region containing the ogt_{ST} gene were sequenced by using the Sequenase sequencing kit, version II (U.S. Biochemical Corp., Cleveland, Ohio) by the dideoxy-chain termination method (44).

The maxicell method of Sancar et al. (43) was used to identify proteins encoded by plasmids pYG617 and pYG601 in CSR603 transformants. Plasmid pYG601 carries the *E. coli ogt* gene in pBluescriptKS+. The proteins were

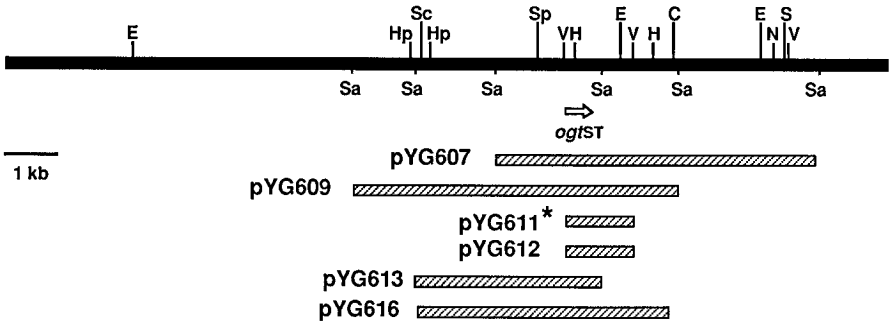

FIG. 1. Physical map of *S. typhimurium* chromosomal DNA in the region of ogt_{ST}. The top line illustrates this map. The hatched bars indicate fragments of DNA cloned or subcloned into plasmid vectors. The open arrow represents the presumed location and direction of transcription of the ogt_{ST} gene. The asterisk indicates that the fragment inserted in pYG611 is the same as that in pYG612 but in the opposite orientation. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nru*I; S, *Sal*I; Sa, *Sau*3AI; Sc, *Sca*I; Sp, *Sph*I; V; *Eco*RV.

labeled with [^{35}S]methionine (New England Nuclear, Boston, Mass.). Samples were run on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel, and the labeled proteins were visualized by fluorography.

Construction of Δogt_{ST} and Δada_{ST}-Δogt_{ST} strains of *S. typhimurium* TA1535. The ogt_{ST} gene of *S. typhimurium* TA1535 was disrupted by the preligation method (60). Plasmid pYG616 (8.1 kb) was digested with two enzymes, *Eco*RI and *Sph*I (Nippon Gene, Tokyo, Japan), and the cohesive ends of the resulting 6.75-kb DNA fragment were converted to blunt ends with a DNA-blunting kit (Takara Shuzo, Kyoto, Japan). A 1.3-kb fragment of pACYC184 carrying the chloramphenicol resistance (Cm^r) gene was ligated to the blunt-ended fragment, and the resulting plasmid was designated pYG620. The plasmid was modified by introduction into *S. typhimurium* LB5000 that lacks three restriction systems, and the modified plasmid was amplified in strain TA1535. The plasmid was digested with *Apa*LI (Takara Shuzo) to remove the replication origin, and the 4.5-kb linear DNA fragment containing the Cm^r gene between the flanking regions of the ogt_{ST} gene was purified with Geneclean kit II (Bio101, La Jolla, Calif.). The purified DNA was treated with T4 DNA ligase and introduced into strain TA1535 by electroporation (60). Chloramphenicol-resistant colonies were selected and examined for deletion of the chromosomal ogt_{ST} gene by Southern hybridization. Strain YG7108, a Δogt_{ST}-Δada_{ST} derivative of TA1535, was also constructed by electroporation of the ligated DNA into the Δada_{ST} strain, YG7100.

Southern hybridization. Bacterial DNA was isolated as described previously (58), and 1 μg of the DNA was digested with *Eco*RI, *Eco*RV, or *Hin*dIII and then subjected to electrophoresis in a 0.8% agarose gel. Blotting of the DNA onto a nylon filter (Hybond-N; Amersham, Amersham, United Kingdom) was carried out by passive diffusion. The DNA fragments used as probes were the 2.95-kb *Eco*RI-*Eco*RI DNA fragment of plasmid pYG603 carrying the *E. coli ada* gene and the 1.5-kb *Hin*dIII-*Hin*dIII DNA fragment of plasmid pYG607 carrying the ogt_{ST} gene. The DNA fragments were labeled with [α-^{32}P]dCTP (New England Nuclear) by using the BcaBEST Labeling Kit (Takara Shuzo). The hybridization was carried out as described previously (51).

Immunoassay of induction of Ada_{ST} and *E. coli* Ada proteins. Exponential cultures (A_{600} of 0.2) of strains AB1157, TA1535, YG7100, YG7104, and YG7108 in LB medium supplemented with 0.2 μg of biotin per ml were treated with 5 μg of MNNG per ml at 37°C for 30 min. The cells (1 ml) were harvested and resuspended in 15 μl of detergent buffer (55). Five or fifteen microliters of cell lysates was resolved by SDS–15% polyacrylamide gel electrophoresis (PAGE), and the proteins were transferred to a nitrocellulose filter. The induced *E. coli* Ada or Ada_{ST} proteins were detected immunologically with two anti-Ada monoclonal antibodies, peroxidase-conjugated rabbit anti-mouse immunoglobulins, and the ECL Western blotting (immunoblotting) detection system (Amersham International), as described previously (46).

Detection of active MTs. Active MT was detected by monitoring the transfer of radioactivity from the ^{3}H-MNU-treated DNA substrate containing O^6-[^{3}H]MeG residues (16 Ci/mol) (13) to a protein in cell extracts. The extracts were prepared as described by Vaughan and Sedgewick (55). Extract containing 100 μg of protein was incubated with the DNA substrate (4,000 cpm) in 18 μl of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.8)–10 mM dithiothreitol–1 mM EDTA at 37°C for 30 min. Eighteen microliters of 2× detergent buffer (55) was added. The ^{3}H-labeled self-methylated MTs were resolved by SDS–12% PAGE and visualized by fluorography. Demethylation of O^6-MeG in the ^{3}H-methylated substrate was assayed as described previously (55).

Fluctuation test. An overnight culture was diluted with fresh LB broth to approximately 10^4 cells per ml. Thirty 3-ml aliquots were grown at 37°C with aeration to an A_{600} of 0.1 to 0.2 (approximately 2×10^8 cells per ml). To determine the cell density more precisely, aliquots (100 μl) from at least three cultures were diluted, plated on LB plates, and incubated overnight at 37°C. To determine the number of His^+ revertants per culture, the cells were harvested by centrifugation and resuspended in medium E salt solution (150 μl). Molten soft agar (2 ml) was added to the suspensions, and the cells were plated on Vogel-Bonner plates containing L-histidine and D-biotin (24). The plates were incubated at 37°C for 2 days, and the number of His^+ revertants was scored.

Mutagenicity assays. The mutagenicity of MNNG was assayed as described previously (60), and the induced-mutation frequency was calculated (31). Briefly, exponential cultures were exposed to MNNG for 5 min before washing and plating of the cells to determine the number of survivors and His^+ revertants. The Ames test with *S. typhimurium* TA1535 and its derivatives was carried out as described by Maron and Ames (24), with preexposure of the cells to the alkylating agents for 20 min at 37°C before plating without removal of the agent. All plates were incubated for 2 days at 37°C before the number of His^+ revertants per plate was determined.

RESULTS

Cloning of ogt_{ST} encoding constitutive MT of *S. typhimurium*. To clone the ogt_{ST} gene, an *S. typhimurium* genomic library was screened for plasmids that conveyed resistance to the cell-killing effects of MNNG to KT233, a Δada-Δogt strain of *E. coli*. We obtained about 1,100 transformants that grew on plates containing MNNG. To confirm that resistance was due to the presence of plasmids carrying *S. typhimurium* chromosomal DNA, the plasmids were isolated from 48 resistant colonies selected randomly, reintroduced into KT233, and confirmed to convey MNNG resistance. Of the 48 plasmid clones, 27 exhibited a physical map very similar to that of the ada_{ST} gene and were excluded from the candidates for the ogt_{ST} gene (data not shown). An additional nine clones were excluded because they hybridized with the ada_{ST} probe but not with the *E. coli ogt* probe in Southern hybridization analysis (data not shown). The remaining 12 clones hybridized with the *E. coli ogt* probe but not with the *E. coli ada* probe (data not shown). These results strongly suggested that the chromosomal DNA segments carried on these 12 plasmids contained the ogt_{ST} gene.

Of the 12 clones which hybridized with the *E. coli ogt* gene, plasmids pYG607 and pYG609 carrying 5.7-kb and 5.9-kb fragments of *S. typhimurium* DNA, respectively, were examined in more detail, and their physical maps were determined (Fig. 1). The overlapping fragments derived from these two plasmids covered a region of about 10 kb of chromosomal DNA. To locate the ogt_{ST} gene more precisely, smaller fragments were subcloned and tested for their ability to complement the MNNG sensitivity of *E. coli* KT233 (Δ*ada ogt*). The *Sca*I-*Cla*I DNA fragment (4.3 kb) derived from the pYG609 was subcloned between the blunt-ended *Eco*RI and *Nru*I sites of pBR322. The resulting plasmid, pYG616, restored resistance to the cytotoxicity of MNNG to the *ada-ogt* mutant of *E. coli*,

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        10          20          30          40          50          60
ACAATGTCAG GCAATTAAGC CGCCTCGCCA GGCTCTCTTT TTCTCCGACT ACGATATTAC

        70          80          90         100         110         120
TGTCGCTAAA TGTGTTATCC CTGACTATCT TTTAAGGAGT ATGGTTGCGG GTATTCCTGG

       130         140         150         160         170         180
CATGATATCT TGTCTCTTAC GTTAGATTAA GACGATGTGA GAGACCGATG CTGAGATTAC
                                                 M   L   R   L   L

       190         200         210         220         230         240
TTGAAGAGAA GATAGCCACG CCATTAGGAC CGTTATGGGT GGTTTGCGAT GAGCAGTTTC
  E   E   K   I   A   T   P   L   G   P   L   W   V   V   C   D   E   Q   F   R

       250         260         270         280         290         300
TACTGCGGGC CATTGAGTGG GAACAGTACC GCGATCGTAT GGAGCAACTG CTAAATATCC
  L   R   A   I   E   W   E   Q   Y   R   D   R   M   P   Q   L   L   N   I   H

       310         320         330         340         350         360
ACTACCGTCA CGAAGGCTAT GAACGCGTTT CTGCGACTAA CCCCGGTGGA CTCAGCGATA
  Y   R   H   E   G   Y   E   R   V   S   A   T   N   P   G   G   L   S   D   K

       370         380         390         400         410         420
AGCTTGCAGA TTATTTTGCA GGCAATCTCG CCGTAATTGA TACCCTGGAA ACCGCCACGG
  L   A   D   Y   F   A   G   N   L   A   V   I   D   T   L   E   T   A   T   G

       430         440         450         460         470         480
GGGGCACACC TTTTCAACGG GAAGTATGGC AGGCATTGCG CGCTATCCCC TGCGGGCAGG
  G   T   P   F   Q   R   E   V   W   Q   A   L   R   A   I   P   C   G   Q   V

       490         500         510         520         530         540
TGATGCACTA TGGTCAACTG GCGGCGCAAC TGGGACGACC GGGCGCCGCA CGCGCAGTCG
  M   H   Y   G   Q   L   A   A   Q   L   G   R   P   G   A   A   R   A   V   G

       550         560         570         580         590         600
GTGCTGCGAA TGGTGCTAAC CCCATCAGTA TTGTTGTTCC CTGCCATCGC GTCATCGGGC
  A   A   N   G   A   N   P   I   S   I   V   V  [P   C   H   R   V] I   G   R

       610         620         630         640         650         660
GTAACGGCAC TCTGACCGGA TACGCAGGCG GCGTGCAGCG AAAAGAGTGG CTATTACGCC
  N   G   T   L   T   G   Y   A   G   G   V   Q   R   K   E   W   L   L   R   H

       670         680         690         700         710         720
ATGAAGGCTA TCTTTTATTA TGAATATACA GGCAAAAAGT GCCTTATCGG TCACACTTTT
  E   G   Y   L   L   L  Stop

       730         740         750         760         770         780
ATGTAAAGCA ACAACAAATA AATTACGTGT TTTCAAATAG ATAAAAATAT TCATCAAACT

       790         800         810         820
TATACTTGAA TTATTCCTTC TCCGGGATAG CTCAGACTTA
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FIG. 2. Nucleotide sequence of ogt_{ST}. The deduced amino acid sequence is shown as single-letter symbols. The putative active site amino acid sequence is boxed.

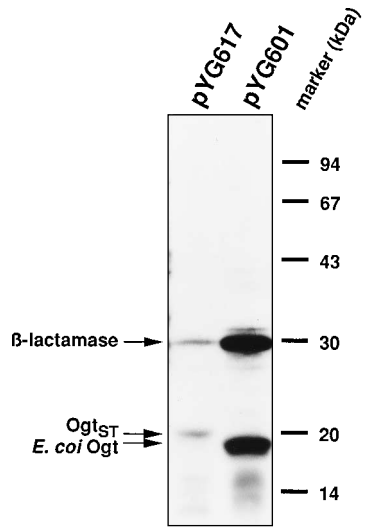

FIG. 3. Autoradiogram of ^{35}S-labeled proteins produced in maxicells of either strain CSR603/pYG617 carrying the ogt_{ST} gene or strain CSR603/pYG601 carrying the *E. coli ogt* gene.

suggesting that the ogt_{ST} gene is located within this 4.3-kb DNA region (Fig. 1). Five of the 12 overlapping clones contained a 1.3-kb *Eco*RV-*Eco*RV fragment, which was subcloned into the *Eco*RV site of pBR322. While plasmid pYG612 carrying the 1.3-kb DNA fragment did not restore MNNG resistance to strain KT233, another plasmid, pYG611, carrying the same 1.3-kb DNA in the opposite direction did (Fig. 1). These results suggested that the coding region of the ogt_{ST} gene was present on this 1.3-kb *Eco*RV-*Eco*RV DNA fragment but that the promoter region was incomplete. In the case of pYG611, the transcriptional direction of ogt_{ST} may have been the same as that of the tetracycline resistance gene into which the *Eco*RV-*Eco*RV DNA fragment was inserted, so that the ogt_{ST} gene was transcribed and expressed from the tetracycline promoter.

DNA sequence of ogt_{ST} and identification of its gene product in maxicells. An 800-bp sequence containing the apparent ogt_{ST} gene and its flanking region was determined on both DNA strands with overlapping sequencing reactions. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 2. The ogt_{ST} coding region lies between nucleotides 168 and 680, beginning with an ATG codon that is downstream of a Shine-Dalgarno sequence at the appropriate position and ending with a TGA codon. The predicted Ogt$_{ST}$ protein consists of 171 amino acid residues, and its estimated molecular mass is 19,006 Da. The estimated molecular mass of the ogt_{ST} gene product identified by the maxicell procedure was 20 kDa (Fig. 3), which agrees closely with the value predicted from the DNA sequence. The 5-amino-acid sequence conserved among Ada-related proteins, Pro-Cys-His-Arg-Val, was also found at amino acid residues 138 to 142 (4, 10, 20, 41). The nucleotide sequence showed 77% identity to the *ogt* gene of *E. coli* (35). The identity at the amino acid level between the two gene products was 88%. These results suggested that the cloned DNA fragment of *S. typhimurium* contained an *ogt*-like gene and the open reading frame shown in Fig. 2 was the coding region of this gene.

Construction of ogt_{ST}- or ada_{ST}-ogt_{ST}-deletion derivatives of *S. typhimurium* TA1535. A 1.4-kb *Sph*I-*Eco*RI DNA fragment carrying the ogt_{ST} gene of plasmid pYG616 was replaced with a 1.3-kb blunt-ended *Hae*II-*Hae*II DNA fragment containing the Cm^r gene derived from pACYC184. The resulting plasmid, pYG620, (Fig. 4) was unable to confer MNNG resistance to strain KT233. The plasmid pYG620 was digested with *Apa*LI, and the resulting 4.5-kb DNA fragment carrying the Cm^r gene flanked by *S. typhimurium* chromosomal DNA was used to disrupt the ogt_{ST} gene of TA1535 and its ada_{ST}-deletion derivative, YG7100, by the preligation method (60).

Disruption of the ogt_{ST} gene was confirmed by Southern blotting analysis using the ogt_{ST} gene as a DNA probe (Fig. 5). When digested with *Eco*RI or *Eco*RV, DNA from the ogt_{ST} deletion strains (YG7104 and YG7108) contained one hybridizing band of 3.0 and 2.3 kb, respectively, whereas DNA from the ogt^+ strain (TA1535) exhibited two bands of 9.4 and 2.7 kb for *Eco*RI and 2.3 and 1.3 kb for *Eco*RV (Fig. 5). *Hin*dIII digests of DNA from the Δogt_{ST} strains showed a single band of 20 kb (Fig. 5, lanes 8 and 9), whereas DNA from the ogt_{ST}^+ strains had a single band of 1.5 kb (Fig. 5, lane 7). These results indicated that the ogt_{ST} gene of the Cm^r colonies was replaced by the Cm^r gene.

Biochemical characterization of ogt_{ST}- and ada_{ST}-ogt_{ST}-deletion derivatives of *S. typhimurium* TA1535. The absence of the Ogt and Ada proteins from crude lysates of the Δ*ogt*-Δ*ada* mutants was verified biochemically. The inducible Ada$_{ST}$ protein was monitored by immunoblotting with two anti-Ada monoclonal antibodies raised against the *E. coli* Ada protein. MNNG induction of the Ada$_{ST}$ protein was readily detected in TA1535 and YG7104 (Δogt_{ST}) although the extent of induction

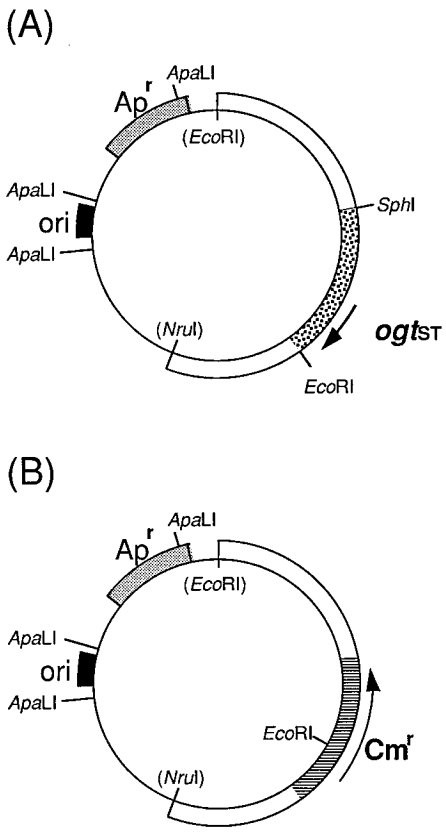

FIG. 4. Physical maps of plasmids pYG616 (A) and pYG620 (B) used for the construction of Δogt_{ST} strains. Filled boxes, replication origins; stippled boxes, genes encoding β-lactamase; unfilled box, chromosomal flanking regions of ogt_{ST}; dotted box, ogt_{ST} gene; striped box, *Hae*II-*Hae*II fragment carrying Cm^r gene.

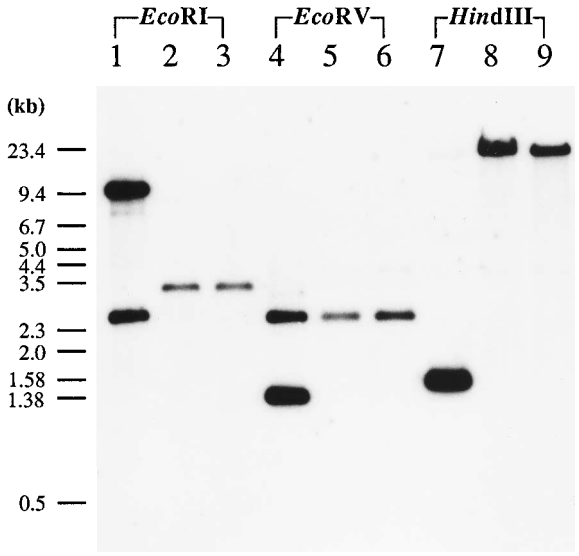

FIG. 5. Southern hybridization analysis for deletion of ogt_{ST} in strains YG7104 and YG7108. Bacterial DNAs were digested with *Eco*RI, *Eco*RV, or *Hin*dIII and run on a 0.8% agarose gel. A 1.5-kb *Hin*dIII-*Hin*dIII DNA fragment containing the ogt_{ST} coding region was labeled with [α-^{32}P]dCTP and used as a probe. Lanes 1, 4 and 7, TA1535; lanes 2, 5 and 8, YG7104 (Δogt_{ST}); lanes 3, 6 and 9, YG7108 (Δada_{ST} Δogt_{ST}).

was severalfold less than that in *E. coli* AB1157, as noted previously (55). The Ada_{ST} protein was not detected in YG7100 (Δada_{ST}) or YG7108 (Δada_{ST} Δogt_{ST}) (Fig. 6A). The constitutive Ogt protein was monitored by incubation of crude extracts with ^{3}H-MNU-treated DNA, and the self-methylated Ogt protein was visualized by fluorography. A strong band corresponding to the 20-kDa Ogt_{ST} protein was observed in TA1535 and YG7100 (Δada_{ST}) but not in YG7104 (Δogt_{ST}) and YG7108 (Δada_{ST} Δogt_{ST}) (Fig. 6B). By this technique, the low basal level of the Ada protein was also detected in uninduced *E. coli* AB1157 as a faint 39-kDa band but was not detected in *E. coli* GW7101 (Δ*ada*). An extremely faint band of Ada_{ST} protein was just visible (certainly in the original autoradiograph) in TA1535 and YG7104 (Δogt_{ST}) (Fig. 6B). These results verify that *S. typhimurium* YG7100, YG7104, and YG7108 lack the Ada_{ST}, the Ogt_{ST}, and the Ada_{ST} plus Ogt_{ST} proteins, respectively. The Ogt protein appeared to be present at very similar levels in *E. coli* AB1157 and *S. typhimurium* TA1535 (Fig. 6B). This was substantiated by assaying the removal of O^6-[^{3}H]MeG from the DNA substrate (13). The activity in AB1157 and GW7101 extracts was 0.4 to 0.5 U/mg of protein, whereas that in TA1535 and YG7100 was 0.5 to 0.7 U/mg of protein. The activity in the *ogt*-deletion derivatives of both *E. coli* and *S. typhimurium* was <0.04 U/mg of protein, the limit of detection of the assay.

Sensitivities of ogt_{ST}- and ada_{ST}-ogt_{ST}-deletion derivatives of TA1535 to MNNG. Sensitivities of strains YG7100, YG7104, and YG7108 to the killing and mutagenic effects of MNNG were compared with those of the parent strain, TA1535. Sensitivity to the killing action of MNNG decreased in the order of YG7108 (Δada_{ST} Δogt_{ST}), YG7100 (Δada_{ST}), and YG7104 (Δogt_{ST}), or TA1535. The resistance of YG7104 was similar to that of the parent strain TA1535: the survival of TA1535 and

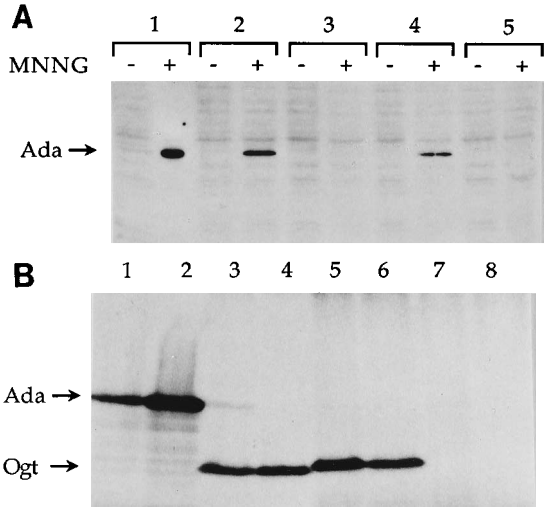

FIG. 6. (A) Recognition of MNNG-inducible proteins by anti-Ada monoclonal antibodies in *E. coli* and *S. typhimurium*. Cultures of *E. coli* AB1157 (lane 1), *S. typhimurium* TA1535 (lane 2), *S. typhimurium* YG7100 (lane 3), *S. typhimurium* YG7104 (lane 4), and *S. typhimurium* YG7108 (lane 5) were treated with (+) or without (−) MNNG (5 μg/ml), and cell lysates (15 μl for each lane, except 5 μl of MNNG-treated AB1157) were analyzed by SDS-PAGE and immunoblotted with two anti-Ada monoclonal antibodies. A chemiluminescence system was used to detect the peroxidase-conjugated secondary antibody. (B) Fluorography of purified Ada protein and crude cell extracts (100 μg of protein) following incubation with ^{3}H-MNU-treated DNA. Lanes: 1, Ada protein (0.03 U) 2, Ada protein (0.3 U) 3, *E. coli* AB1157; 4, *E. coli* GW7101; 5, *S. typhimurium* TA1535; 6, *S. typhimurium* YG7100; 7, *S. typhimurium* YG7104; 8, *S. typhimurium* YG7108.

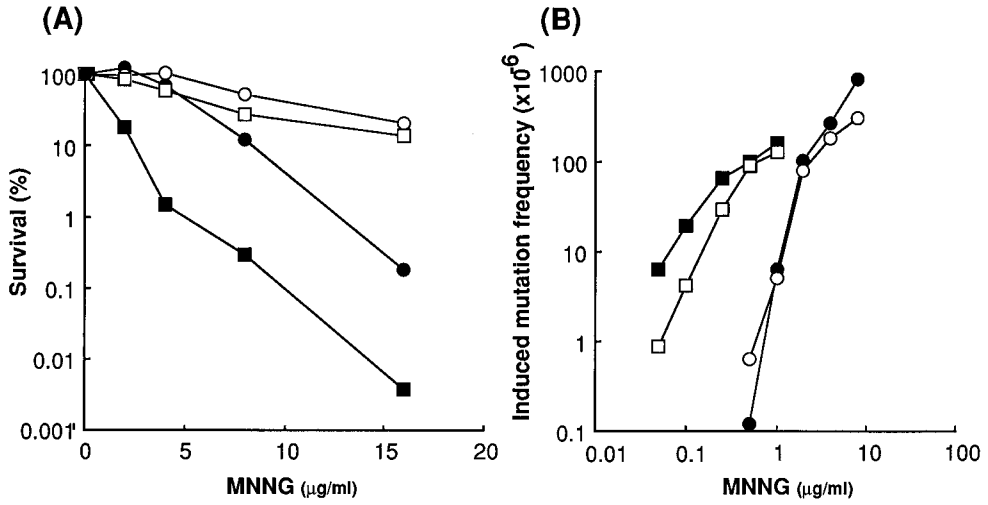

FIG. 7. Sensitivity of ogt_{ST}-deletion mutants of *S. typhimurium* to the lethal (A) and mutagenic (B) effects of MNNG. Exponential liquid cultures were exposed to MNNG for 5 min at 37°C. ○, TA1535 (ada_{ST}^{+} ogt_{ST}^{+}); ●, YG7100 (Δada_{ST}); □, YG7104 (Δogt_{ST}); ■, YG7108 (Δada_{ST} Δogt_{ST}).

YG7104 was about 20% at an MNNG concentration of 16 μg/ml whereas those of YG7100 and YG7108 were 0.2 and 0.004%, respectively, at this concentration (Fig. 7A). In contrast, YG7104 was hypersensitive to the mutagenic action of MNNG, whereas strain YG7100 (Δada_{ST}) had a level of sensitivity similar to that of TA1535.(Fig. 7B). In addition, the ada_{ST}-ogt_{ST}-deletion strain, YG7108, was slightly more sensitive than YG7104 (Δogt_{ST}). These results suggested that in *S. typhimurium* the two MTs, Ada_{ST} and Ogt_{ST}, play different roles and protect cells against the killing and mutagenic effects of alkylating agents.

Spontaneous mutagenesis of ogt_{ST}-deletion strains. The frequency of spontaneous mutagenesis of the Δada_{ST} strain YG7100 was not detectably different from that of TA1535. However, the Δogt_{ST} strains, YG7104 and YG7108, exhibited two to three times higher spontaneous mutation frequencies than the ogt_{ST}^{+} strains, TA1535 and YG7100 (Fig. 8). Interestingly, the Δada_{ST}-Δogt_{ST} strain YG7108 exhibited a slightly, but significantly, higher spontaneous mutation frequency than strain YG7104 (Δogt_{ST}). The mean numbers of the spontaneous mutants per plate after 2 days of incubation were 7.4 for TA1535, 7.5 for YG7100, 18.4 for YG7108, and 13.8 for YG7104. These results suggest that the Ogt_{ST} protein plays a major role in protecting *S. typhimurium* from endogenously generated alkylating agents and also that the Ada_{ST} protein contributes to this protection when the Ogt_{ST} protein is absent.

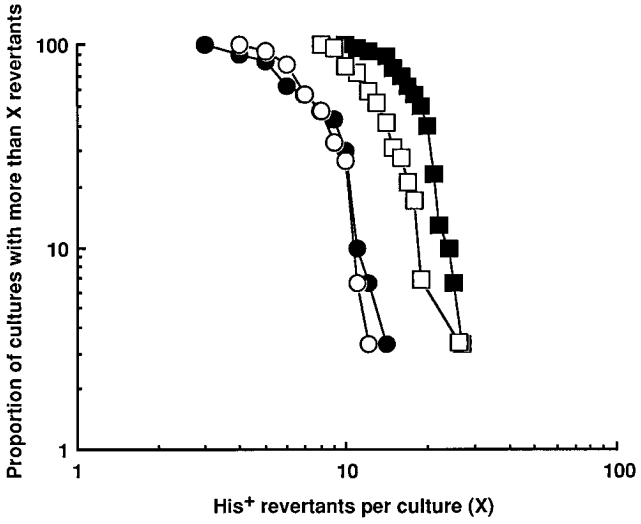

FIG. 8. Spontaneous mutation rates. The proportion of culture generating more than *X* revertants per plate versus the number of revertants per plate (X) was plotted. ○, TA1535 (ada_{ST}^{+} ogt_{ST}^{+}); ●, YG7100 (Δada_{ST}); □, YG7104 (Δogt_{ST}); ■, YG7108 (Δada_{ST} Δogt_{ST}).

***S. typhimurium* YG7104 and YG7108 are highly sensitive to simple alkylating agents.** To further characterize the newly constructed ogt_{ST}-deletion strains YG7104 (Δogt_{ST}) and YG7108 (Δada_{ST} Δogt_{ST}), we examined their sensitivities to mutagenic alkylating agents that have long alkyl groups, such as ethyl, propyl, and butyl groups (Fig. 9). Strains YG7104 and YG7108 were more sensitive to MNNG, ENNG, PNNG, and BNNG than was the parent strain, TA1535. They were strikingly sensitive to ENNG in the dose range examined, which did not produce any His^{+} revertants in TA1535. The sensitivities of YG7108 to ENNG, PNNG, and BNNG were almost the same as those of YG7104. For MNNG, however, YG7108 exhibited a slightly but significantly higher degree of sensitivity than YG7104 at doses less than 0.5 μg per plate. These results suggest the Ogt_{ST} protein plays a major role in protecting cells from the mutagenic effects of all the alkylating agents tested, whereas the Ada_{ST} protein has a detectable role in protecting against low doses of MNNG only when the Ogt_{ST} protein is absent. The extreme sensitivity of ogt_{ST} mutants to ENNG may suggest that the Ogt_{ST} protein is particularly efficient in repairing toxic ethylation damage.

DISCUSSION

In a previous paper, we reported that in contrast to *E. coli ada* mutants, *S. typhimurium* YG7100, an ada_{ST}-deletion derivative of TA1535, was not sensitive to the mutagenic action of MNNG (47, 60). This observation suggested that unlike the *E. coli* Ada protein, the *S. typhimurium* Ada protein does not play a major role in protecting cells from the mutagenicity of methylating agents. *E. coli* has a second MT, the Ogt protein, with a molecular mass of 19 kDa. *S. typhimurium* also possesses a 19-kDa protein that can transfer methyl groups from methylated DNA to itself (35, 38, 40). These results led us to postu-

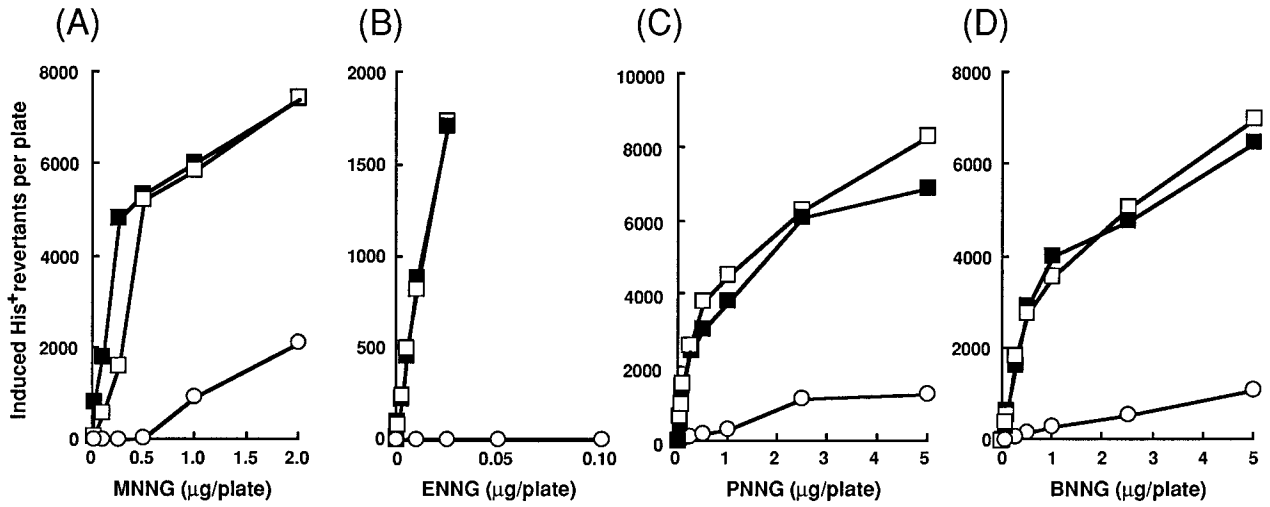

FIG. 9. Mutagenic responses of *S. typhimurium* TA1535 and its Δogt_{ST}-Δada_{ST} derivatives to simple alkylating agents in the Ames mutagenicity test. (A) MNNG; (B) ENNG; (C) PNNG; (D) BNNG. ○, TA1535 (ada_{ST}^{+} ogt_{ST}^{+}); □, YG7104 (Δogt_{ST}); ■, YG7108 (Δada_{ST} Δogt_{ST}).

late that *S. typhimurium* might possess an Ogt_{ST} protein, which plays a central role in protecting cells from mutagenic methylating agents. In this paper, we have described the cloning, sequencing, and disruption of the ogt_{ST} gene of *S. typhimurium*. The ogt_{ST} gene was cloned from a plasmid library of *S. typhimurium* TA1538 DNA as a gene that conveys MNNG resistance to an *ada-ogt* mutant of *E. coli*. DNA sequence analysis indicated that the cloned gene encoded a protein with a molecular mass of 19 kDa, and this was verified by the maxicell technique (Fig. 3). The DNA sequence of the open reading frame showed 77% identity to the *E. coli ogt* gene (35) at the nucleotide level and included a consensus active site sequence of Pro-Cys-His-Arg-Val found in *ada*-related proteins (4, 8, 10, 41) (Fig. 2). Thus, we conclude that the cloned gene is a homolog of the *E. coli ogt* gene and refer to this gene as ogt_{ST}.

In order to construct ogt_{ST}- and ogt_{ST}-ada_{ST}-deletion derivatives of *S. typhimurium* TA1535, we employed the preligation method which we have developed recently (60). Linear DNA fragments carrying a drug resistance gene inserted between the flanking chromosomal regions of the ogt_{ST} gene were concatemerized by ligation before introduction into *S. typhimurium* TA1535 or YG7100 (Δada_{ST}). Of eight Cm^r derivatives of strain TA1535, one (about 13%) was a true gene replacement, in which the ogt_{ST} gene on the chromosome was replaced by the Cm^r gene (Fig. 5). Similarly, about 22% of the Cm^r derivatives of strain YG7100 (4 of 18) were true gene replacements. Crude cell extracts of the ogt_{ST}-deletion derivatives did not exhibit any detectable Ogt_{ST} protein, when monitored for self-methylation on incubation with ^{3}H-MNU-treated DNA (Fig. 6B). Since *S. typhimurium* YG7108, an ogt_{ST}-ada_{ST}-deletion derivative of strain TA1535, did not have any detectable O^6-MeG DNA MT activities, Ogt_{ST} and Ada_{ST} are probably the only two such MTs in *S. typhimurium*. The constitutive *E. coli* Ogt protein is present at about 30 molecules per cell (28), and the Ogt_{ST} protein appeared to occur at a similar level in *S. typhimurium* (Fig. 6B).

S. typhimurium YG7104, (Δogt_{ST}) was more sensitive to mutagenesis by MNNG than the parent strain TA1535 (Fig. 7) and also had a two to three times higher spontaneous mutation frequency (Fig. 8). Strain YG7100 (Δada_{ST}) did not detectably differ in these characteristics from the wild type. These results are in contrast to the reported responses of the *ogt* and *ada* mutants of *E. coli*: a Δ*ogt* mutant of *E. coli* AB1157 has a level of mutability by MNNG similar to that of the parent strain, whereas a Δ*ada* mutant is hypersensitive. Thus, in *E. coli* the Ada protein plays a major role in protecting cells from mutagenic methylation damage (39, 53), whereas in *S. typhimurium* Ogt_{ST} has this role and protects *S. typhimurium* from the mutagenicity of both endogenous and exogenous methylating agents. The ogt_{ST}-encoded MT is probably the constitutive O^6-MeG DNA repair activity first observed in *S. typhimurium* TA1535 by Guttenplan and Milstein (7). It must be noted, however, that the Ada_{ST} protein appeared to contribute to a small but significant extent to the protection in *S. typhimurium* when the Ogt_{ST} protein was absent, because YG7108 (Δada_{ST} Δogt_{ST}) was slightly more sensitive to MNNG and had a slightly higher spontaneous mutation frequency than YG7104 (Δogt_{ST}) (Fig. 8 and 9A). The ada_{ST} gene can be induced by exposure of cells to a low concentration of MNNG although the extent of induction is much smaller than that of the *E. coli ada* gene (8, 55) (Fig. 7A). When *S. typhimurium* is exposed to methylating agents and Ogt_{ST} is consumed by self-methylation during DNA repair (1), the induced Ada_{ST} protein may serve a small backup role in protecting cells from mutagenic methylation damage.

Since the ada_{ST}-deletion derivative of strain TA1535 is hypersensitive to the killing effect of MNNG (60) (Fig. 7A), the ada_{ST} gene of *S. typhimurium* appears to play a major role in protecting cells from toxic methylation damage (Fig. 7A). In fact, the ada_{ST} gene was initially isolated by its ability to complement the lethal effects of MNNG on an *E. coli ada* mutant. This protection is unlikely to result from the repair of O^6-MeG in DNA because the Δogt_{ST} mutant, which has a greater deficiency in MT activity than the Δada_{ST} mutant, is not sensitive to MNNG killing. A more likely explanation is that the Ada_{ST} protein can induce expression of the *E. coli alkA* gene encoding 3-methyladenine DNA glycosylase that excises toxic 3-methyladenine from DNA (20). Studies of mutant *E. coli* Ada proteins have suggested that different regulatory mechanisms are involved in the induction of the *alkA* and *ada* genes (48). By analogy to these mutants, we suggest that the ada_{ST}-encoded protein may have different abilities to function as a transcriptional activator of the ada_{ST} and $alkA_{ST}$ genes of *S. typhimurium*: methylated Ada_{ST} protein may efficiently induce ex-

pression of the $alkA_{ST}$ gene but, as previously reported, be a weak inducer of the ada_{ST} gene itself (8, 55). In other words, the Ada_{ST} protein may be a naturally occurring mutated Ada protein.

Alkylating agents as well as active oxygen species are regarded as endogenous genotoxic agents. *S*-adenosylmethionine is a weak alkylating agent and can nonenzymatically methylate protein and DNA (18). *E. coli ada-ogt* double mutants have a higher rate of spontaneous mutation than the wild-type or the single mutants, and this increased mutation frequency occurs in both dividing and nondividing cells (23, 39). In this study, we have demonstrated that ogt_{ST}-ada_{ST}- and single ogt_{ST}-deletion derivatives of *S. typhimurium* have high rates of spontaneous mutation in dividing cells. Since mutagenesis was monitored in these strain by reversion of *hisG46*, which mainly occurs by GC-to-AT transition mutations, we suggest that an endogenous mutagen(s) which induces these transitions is generated in actively dividing cells. This is consistent with the report that the spontaneous rate of GC-to-AT transitions as well as GC-to-CG transversions was elevated about fourfold in the *E. coli ada-ogt* double mutant during exponential growth (23). Potential candidates for such endogenous DNA-methylating agents include *S*-adenosylmethionine, nitrosated amines, and methyl radicals generated by lipid peroxidation (18).

S. typhimurium TA1535 is used as the tester strain in the Ames mutagenicity test to detect environmental mutagens and carcinogens. The work described in this paper raises the interesting possibility that the ogt_{ST}-deletion (YG7104) and the ogt_{ST}-ada_{ST}-deletion (YG7108) derivatives of strain TA1535 could be used as tester strains that are highly sensitive to mutagenic alkylating agents. Strains YG7104 and YG7108 exhibited no threshold responses to methylating, ethylating, propylating, and butylating agents and produced His^+ revertants at very low doses (Fig. 9). In particular, the strains exhibited very high sensitivities to ENNG (Fig. 9B). They were also more sensitive to methyl methanesulfonate than TA100, a pKM101-harboring derivative of strain TA1535 generally used in the Ames test to enhance sensitivity (unpublished results).

Recently, human homologs of DNA mismatch-repair genes (*hMLH2* and *hMSH1*) were cloned and implicated as the critical defective genes involved in colon carcinogenesis (5, 17). Interestingly, some of the mismatch-repair-deficient human cells are more resistant to the killing effect of MNNG than the mismatch repair-proficient cells when MTs are inactivated (2). Thus, Karran and Bignami suggested that mismatch-repair-deficient cells would be selected out if cells lacking MT (because of its self-methylation or an inactivating mutation) were constantly exposed to methylating agents (12). It is suggested that colon cells may be frequently exposed to methylating agents. The newly constructed *Salmonella* strains YG7104 and YG7108 could be used to aid the identification of such methylating agents that might be involved in colon carcinogenesis in human beings.

ACKNOWLEDGMENTS

We thank Mutsuo Sekiguchi for providing the *E. coli* KT233 strain and Yuji Kohara for the λ phage lysate. We thank Keiko Matsui and Shau-Fan Ding for skilled technical assistance and Pietro Taverna for providing information on the fluctuation test.

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