New Method for Gene Disruption in *Salmonella typhimurium*: Construction and Characterization of an *ada*-Deletion Derivative of *Salmonella typhimurium* TA1535

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A new method for gene disruption in *Salmonella typhimurium* was developed. The key steps of this method are to produce restriction fragments with compatible ends, preligate to produce concatemers, and then transform by electrotransformation. We developed and used this method to construct a mutant of *S. typhimurium* TA1535 in which the resident *ada*-like (ada_{ST}) gene was replaced with a kanamycin resistance gene to produce an ada_{ST}-deletion mutant derivative. The *S. typhimurium* ada_{ST}-deletion strain did not exhibit a higher level of mutability upon treatment with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine than did its wild-type parent strain. However, it did exhibit a higher sensitivity with respect to killing by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. The ability of Ada_{ST} to function as a transcriptional activator is discussed.

Escherichia coli cells have the ability to acquire increased resistance to the lethal and mutagenic effects of alkylating agents during exposure of cells to sublethal concentrations of methylating and ethylating agents (14, 33). This phenomenon has been termed the adaptive response and requires a functional *ada* gene, which encodes a 39-kDa O^6-methylguanine DNA methyltransferase (MT) (16, 34, 41). This enzyme transfers a methyl group from methylated DNA to its Cys-69 and Cys-321 residues in a stoichiometric fashion (4, 18, 22–24, 28, 40). Methylation of Ada at the Cys-69 residue converts it into an efficient transcriptional activator of the *ada alkB* operon, the *alkA* gene, and the *aidB* gene (17, 25, 36, 40, 42, 45). Thus, the Ada protein plays an important role in the adaptive response, not only as a DNA repair enzyme but also as a transcriptional activator (19). In contrast to the case with *E. coli*, the adaptive response does not seem to occur in the closely related species *Salmonella typhimurium* (8, 19). Recently, we have cloned the *ada*-like gene (ada_{ST}) of *S. typhimurium* TA1538 (10). The gene product, Ada_{ST}, shows 75% similarity at the amino acid level to the Ada protein of *E. coli* and has the two conserved cysteine residues, i.e., Cys-68 and Cys-320. In fact, the Ada_{ST} protein accepts methyl groups from methylphosphotriesters and O^6-methylguanine in DNA (43). Although it has been suggested that the lack of the adaptive response in *S. typhimurium* is due to a low ability of methylated Ada_{ST} to act as a transcriptional activator (10), the exact roles of Ada_{ST} in protecting *S. typhimurium* from the mutagenic and cell-killing effects of alkylating agents have not been thoroughly investigated.

The most convincing method to assess the roles of ada_{ST} is to construct and characterize an ada_{ST}-deletion strain of *S. typhimurium*. In *E. coli*, specific gene disruption can be achieved by a number of methods (3, 9, 13, 15, 32). For example, one approach has been to introduce linearized DNA fragments into a *recB recC sbcB* strain or a *recD* strain of *E. coli* (35, 48). Gene replacement can be detected by drug

resistance if a drug resistance gene is inserted in place of the target gene and flanked by chromosomal DNA from both sides of the target gene. This DNA must also lack a replication origin. Although the strains are proficient in homologous recombination, the mutations of *recB recC* or *recD* inactivate exonuclease V, which digests linear DNA fragments (38). Thus, the introduced linear DNA fragments are relatively stable, thereby increasing the chance of homologous recombination between the DNA fragment containing the deletion and drug resistance marker and its chromosomal homolog. Although these methods are effective in *E. coli*, they are not easily applicable to *S. typhimurium* because there are no *recB recC sbcB* mutant strains of *S. typhimurium* and the *recD* strains are not as widely distributed as the *E. coli recD* mutants.

The Ames *Salmonella* tester strains have additional problems which require the development of alternative methods for gene replacement. Even if mutants deficient in exonuclease V activities were available for *S. typhimurium*, the deletion phenotype could not be transferred to *S. typhimurium* TA1535 by transduction since phage P22 cannot be adsorbed by this strain. P22 adsorption is blocked by the *rfa* mutation, which increases permeability to chemical mutagens (21). *S. typhimurium* TA1535 is an Ames tester strain that is sensitive to the mutagenic effects of alkylating agents.

In this paper, we report a new, simple method for specific gene disruption in *S. typhimurium*. A feature of this method is to treat the linearized DNA fragments having no origin of DNA replication with T4 DNA ligase prior to introducing them into *S. typhimurium*. This pretreatment presumably results in concatemer formation which allows the recombination to occur prior to the degradation by exonuclease V. Since this technique does not require any special mutants or plasmids, it could be applied to other microorganisms. Using this "preligation method," we constructed a derivative of *S. typhimurium* TA1535 in which the chromosomal ada_{ST} gene was replaced with a kanamycin resistance (Km^r) gene. Characterization of the ada_{ST}-deletion derivative of *S. typhimurium* TA1535 suggested that the Ada_{ST} protein plays an important role in protecting cells from the cell-killing effects

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source
E. coli		
AB1157	F⁻ *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE37*	G. C. Walker
GW7101	As AB1157 but *ada-25* Cm^r	G. C. Walker
S. typhimurium		
TA1535	As LT2 wild-type strain but *hisG46* Δ(*gal bio chl uvrB*) *rfa*	B. N. Ames
YG7100	As TA1535 but the *ada*$_{ST}$ gene is replaced with the Km^r gene	This study
YG7101	As TA1535 but has the Km^r gene plus the flanking chromosome at the 3' downstream side of the *ada*$_{ST}$ gene on the chromosome	This study
YG7102	As YG7101 but has multiple copies of the Km^r gene plus the flanking chromosome at the 3' downstream side of the *ada*$_{ST}$ gene on the chromosome	This study
YG7103	As TA1535 but has the Km^r gene plus the flanking chromosome at the 5' upstream side of the *ada*$_{ST}$ gene on the chromosome	This study
Plasmids		
pUC-4K	Plasmid for Km^r gene cartridge; Ap^r	Pharmacia
pYG7001	As pBR322 but has a 5.5-kb fragment of TA1538 DNA carrying the *ada*$_{ST}$ gene; Ap^r	A. Hakura
pYG7510	As pYG7001 but has the Km^r gene instead of the *ada*$_{ST}$ gene; Ap^r	This study

but not from the mutagenic effects of methylating agents in *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used are described in Table 1.

Media and chemicals. LB broth and agar were used for routine bacterial culture and supplemented, when necessary, with 50 μg of ampicillin per ml for plasmid selection and maintenance. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine were supplied by M. Nakadate, National Institute of Hygienic Sciences, Tokyo, Japan. *N*-Methyl-*N*-nitrosourea (MNU) and *N*-ethyl-*N*-nitrosourea were purchased from Sigma Chemical Co., St. Louis, Mo. Semienriched-medium agar plates used for the reversion assay of *argE3* to Arg⁺ in *E. coli* were prepared as previously described (27). Vogel-Bonner minimal agar plates and top agar used for the reversion assay of *hisG46* to His⁺ in *S. typhimurium* TA1535 were prepared as previously described (21).

Preligation method. Plasmid pYG7001 carrying *ada*$_{ST}$ was partially digested with *Eco*RV to remove a 1.6-kb *Eco*RV-*Eco*RV fragment carrying the *ada*$_{ST}$ gene. The resulting 8.3-kb DNA was purified with a Geneclean II kit (Bio 101, La Jolla, Calif.). The purified DNA was ligated with the 1.3-kb *Hinc*II-*Hinc*II DNA fragment carrying the Km^r gene prepared from plasmid pUC-4K (Pharmacia, Inc., Piscataway, N.J.) (44). The resulting plasmid, designated pYG7510, was introduced into *S. typhimurium* TA1535 for modification of DNA. The modified DNA (40 μg) was digested with 120 U of *Apa*LI (Takara Shuzo, Kyoto, Japan), and the 7.8-kb linear DNA fragment carrying the Km^r gene plus the flanking region was purified. The purified DNA (30 μg) was resuspended in water (200 μl), and 1/10 of the purified DNA was treated with T4 DNA ligase by using a DNA ligation kit (Takara Shuzo) in a total volume of 200 μl overnight at 16°C. After heat inactivation of T4 DNA ligase for 5 min at 65°C, the treated DNA was precipitated with ethanol and resuspended in water. The DNA was introduced into competent cells of strain TA1535 by using the Gene Pulser system (set voltage, 2.5 kV; capacitor, 25 μF; electrode gap, 0.2 cm) (Bio-Rad Laboratories, Richmond, Calif.) (5). Competent cells of TA1535 were prepared by the method recommended by Bio-Rad (5), and an aliquot (60 μl) from a suspension of the competent cells was used for each trans-

formation. After addition of 1 ml of warm LB broth, the cells were incubated for 1 h at 37°C. The cells were then poured onto five LB plates containing kanamycin (25 μg/ml) and incubated overnight at 37°C for selection of Km^r colonies.

Southern hybridization analysis. Bacterial DNA was isolated as described previously (47). The DNA (1 μg) was digested with *Sma*I, *Eco*RV, or *Hind*III, and the digested DNA samples were run on a 0.8% agarose gel. DNA in the gel was denatured in situ and transferred to a nylon filter (Hybond-N; Amersham-Japan, Tokyo). Hybridization and washing were performed as described previously (27, 39). The DNA fragments used for DNA probes were labeled with [α-^{32}P]dCTP (DuPont, NEN Research Products, Boston, Mass.) by using the random primer labeling kit (Takara Shuzo).

Alkylation-induced cell killing and mutation. An aliquot (1 ml) of a log-phase culture was mixed with various concentrations of the methylating or ethylating agents dissolved in dimethyl sulfoxide (20 μl) and incubated for 5 min at 37°C with shaking. The treated cells were washed twice with cold saline and resuspended (1 ml). A portion (0.1 ml) of the cells was spread on semienriched-medium agar plates (for *E. coli*) or spread on Vogel-Bonner agar plates (for *S. typhimurium*). Counting of the surviving cells and mutants was carried out as described previously (10). The induced-mutation frequency was calculated as described previously (27).

RESULTS

Treatment of the linear DNA fragments with T4 DNA ligase enhanced gene replacement in *S. typhimurium*. To investigate the in vivo roles of Ada$_{ST}$ protein in *S. typhimurium*, we constructed a mutant strain deficient in this DNA repair protein. Since we have already cloned the *ada*$_{ST}$ gene into plasmid pYG7001 (10), we constructed an *ada*$_{ST}$-disruption mutant of *S. typhimurium* by using this plasmid. The 1.6-kb *Eco*RV-*Eco*RV DNA region of pYG7001, which carries the *ada*$_{ST}$ gene, was replaced in vitro with the 1.3-kb DNA fragment carrying the Km^r gene (Fig. 1). After digestion of the resulting plasmid, pYG7510, with *Apa*LI, the 7.8-kb linear DNA fragment containing the Km^r gene flanked by *Salmonella* chromosomal DNA on either side was purified. The enzyme *Apa*LI was chosen because it was suitable to separate the target sequence from the origin of plasmid replication.

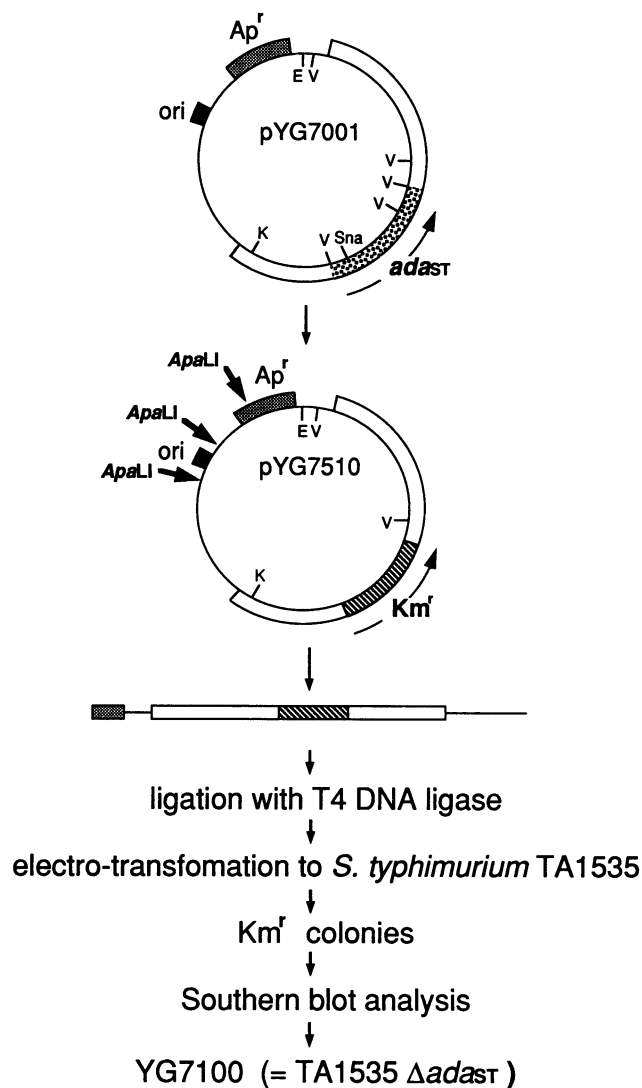At first, we tried to directly introduce the linear DNA

ligation with T4 DNA ligase

electro-transfomation to *S. typhimurium* TA1535

Km^r colonies

Southern blot analysis

YG7100 (= TA1535 Δ*ada*ST)

FIG. 1. Strategy for ada_{ST} gene disruption in *S. typhimurium*. The plasmid pYG7001 was partially digested with *Eco*RV, and the 1.6-kb *Eco*RV-*Eco*RV DNA fragment (▨) carrying the ada_{ST} gene was replaced with the Km^r gene. The resulting plasmid, pYG7510, was digested with *Apa*LI, and the linear DNA fragment carrying the Km^r gene flanked by the chromosomal DNA on either side was purified. After treatment of the DNA with T4 DNA ligase, the DNA was introduced into *S. typhimurium* TA1535 by electrotransformation and Km^r colonies were selected. ori, DNA replication origin; Ap^r, ampicillin resistance gene; Km^r, kanamycin resistance gene. The thin arrows indicate the transcriptional directions of the genes. The thick arrows indicate the restriction sites of *Apa*LI. Restriction enzymes: E, *Eco*RI; V, *Eco*RV; K, *Kpn*I; Sna, *Sna*BI.

fragments into strain TA1535 by electrotransformation (Table 2). Although we increased the amount of DNA to up to 2.5 μg per transformation, no Km^r colonies were obtained. Since exonuclease V activity is the main barrier for efficient gene replacement with linear DNA fragments in *E. coli*, we postulated that multimer formation induced by treatment of the linear DNA fragments with T4 DNA ligase might protect the DNA from exonuclease digestion in *S. typhimurium* and hence increase the frequency of gene replacement. Thus, we treated the purified linear DNA fragments with T4 DNA ligase and introduced the treated DNA into strain TA1535 by

TABLE 2. Efficiency of transformation of *S. typhimurium* TA1535 with linear DNA fragments with or without T4 DNA ligase treatment

DNA per transformation (μg)	No. of Km^r colonies[a]		
	Electrotransformation		Chemical transformation (treated)
	Untreated	Treated	
0.1	0	142	0
0.5	0	1,213	0
2.5	0	359	27

[a] Total number of Km^r colonies per transformation. Transformation efficiencies for electrotransformation and chemical transformation are 4×10^6 and 3×10^3 ampicillin-resistant colonies, respectively, per μg of pYG7510 DNA. Electrotransformation was carried out as described in Materials and Methods; chemical transformation was carried out as described in reference 46. Treated and untreated mean that the DNA was introduced into *S. typhimurium* with or without treatment with T4 DNA ligase, respectively.

electrotransformation. Examination of the preligation products on gels indicated the conversion of the restriction fragment to high-molecular-weight DNA which was greater than 3 to 5 times the size of the desired restriction fragment. By using the ligated DNA fragments, more than 100 Km^r colonies were obtained with 0.1 μg of DNA per transformation, and more than 1,000 Km^r colonies were obtained with 0.5 μg of DNA. When we introduced the treated DNA into strain TA1535 by chemical transformation using calcium chloride, about 30 Km^r colonies were obtained with 2.5 μg of DNA per transformation.

In order to eliminate the possibility that these Km^r colonies were due to undigested plasmid pYG7510 contaminating the preparation used for transformation, we checked the ampicillin sensitivities of 100 colonies obtained and confirmed that all the colonies were sensitive to ampicillin. We also determined that 12 of these ampicillin-sensitive colonies actually had no plasmids by examining cell lysates prepared by the method of Birnboim and Doly (2) by agarose gel electrophoresis followed by ethidium bromide staining. From these results, we concluded that most, if not all, of the Km^r colonies were due to integration of the DNA fragments containing the Km^r gene into the chromosome of *S. typhimurium*.

Structural analyses of the Km^r derivatives of *S. typhimurium* TA1535. In order to characterize the Km^r derivatives of strain TA1535, we determined the sensitivities of the derivatives to the cell-killing effects of MNNG. Of 17 derivatives tested, 7 derivatives exhibited much higher levels of sensitivity to the killing effects of MNNG than did the parent strain TA1535, while the other 10 derivatives exhibited almost the same levels of sensitivity (Fig. 2). In order to determine the molecular basis of the difference in the sensitivity to MNNG, *Sma*I digests of the chromosomal DNAs extracted from 17 Km^r derivatives were examined by Southern hybridization analysis using a probe containing the Km^r gene. From this analysis, the Km^r derivatives were classified into four groups, depending upon their banding patterns (Fig. 2A). All seven derivatives which exhibited higher sensitivities to the killing effects of MNNG showed two bands at 5 and 8 kb (group A). Nine derivatives, which exhibited almost the same sensitivities to MNNG as did the parent strain, showed two bands at 8 and 13 kb (group B). Of the nine derivatives of group B, one derivative exhibited a third intense band at 7.8 kb along with the bands at 8 and 13 kb (group B'). The remaining one derivative showed two
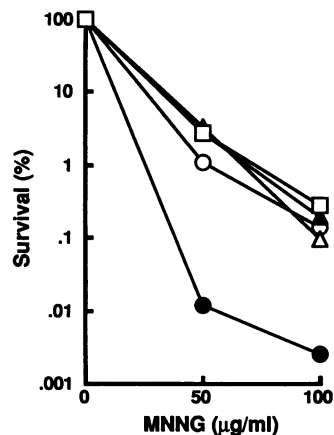
(A)
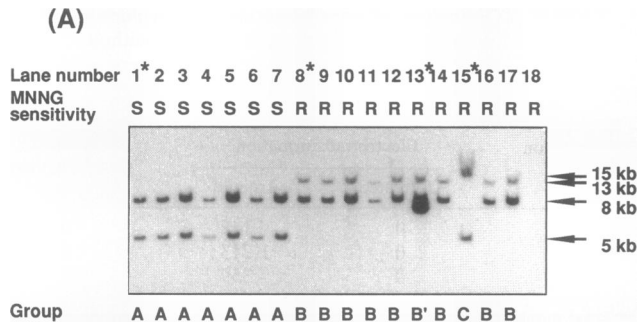
(B)

FIG. 2. (A) Southern hybridization analyses of chromosomal DNAs of the Km^r derivatives of *S. typhimurium* TA1535. The DNAs were digested with *SmaI* and run on a 1% agarose gel. The 1.3-kb DNA fragment carrying the Km^r gene prepared from pUC-4K was labeled with [α-^{32}P]dCTP and used as a probe. R, derivatives that exhibited more than 0.5% survival after the treatment with MNNG (50 μg/ml); S, derivatives that exhibited less than 0.5% survival. The sensitivities of the derivatives to killing by MNNG were measured as described in Materials and Methods. The 17 Km^r derivatives were classified into four groups based on the banding patterns and the sensitivities to MNNG. A representative of each group (*) was used for further analysis: lane 1, *S. typhimurium* YG7100 (group A); lane 8, *S. typhimurium* YG7101 (group B); lane 13, *S. typhimurium* YG7102 (group B'); lane 15, *S. typhimurium* YG7103 (group C). DNA of *S. typhimurium* TA1535 was run in lane 18. (B) Survival curves of the Km^r derivatives of *S. typhimurium* TA1535 with MNNG. ○, *S. typhimurium* TA1535; ●, *S. typhimurium* YG7100 (group A); ▲, *S. typhimurium* YG7101 (group B); □, *S. typhimurium* YG7102 (group B'); △, *S. typhimurium* YG7103 (group C).

bands at 5 and 15 kb (group C). The DNA of strain TA1535 did not hybridize with the Km^r gene, as expected.

To further investigate the detailed structures of the Km^r derivatives, we picked one Km^r derivative each from groups A, B, and C, and *Eco*RV- or *Hind*III digests of their DNAs were analyzed by Southern blotting using a probe carrying a 1.7-kb *Sna*BI-*Kpn*I chromosomal DNA containing part of the *ada*$_{ST}$ gene (Fig. 3). The digests of DNA of strain YG7100, a derivative of group A, exhibited banding patterns different from those of DNA of the parent strain TA1535. Typically, the *Eco*RV-digested DNA of YG7100 exhibited a single band with a molecular size of 4.2 kb, while the DNA of TA1535 exhibited two prominent bands with molecular
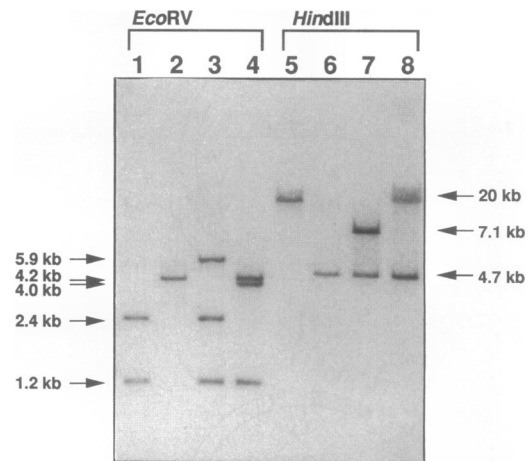

FIG. 3. Southern hybridization analyses of chromosomal DNAs of *S. typhimurium* TA1535 and its Km^r derivatives. The DNAs were digested with *Eco*RV or *Hind*III and run on a 1% agarose gel. The 1.7-kb *Kpn*I-*Sna*BI DNA fragment containing part of the *ada*$_{ST}$ gene prepared from plasmid pYG7001 was labeled with [α-^{32}P]dCTP and used as a probe. Lanes 1 and 5, *S. typhimurium* TA1535; lanes 2 and 6, *S. typhimurium* YG7100 (group A); lanes 3 and 7, *S. typhimurium* YG7101 (group B); lanes 4 and 8, *S. typhimurium* YG7103 (group C).

sizes of 2.4 and 1.2 kb. The *Hind*III-digested DNA of YG7100 exhibited a single band with a molecular size of 4.7 kb, while the DNA of TA1535 exhibited a band whose molecular size was about 20 kb. From these results together with those shown in Fig. 2A, we concluded that the *ada*$_{ST}$ gene of YG7100, and probably those of the other six Km^r derivatives of group A, was replaced exactly with the Km^r gene (Fig. 4, Type A).

The digests of DNA of strain YG7101, a Km^r derivative of group B, exhibited banding patterns different from those of strain YG7100 as well as from those of strain TA1535 (Fig. 3). The DNA of strain YG7101 digested with *Eco*RV exhibited three bands with molecular sizes of 5.9, 2.4, and 1.2 kb, while the DNAs of YG7100 and TA1535 exhibited a single band at 4.2 kb and two bands at 2.4 and 1.2 kb, respectively. The DNA of strain YG7101 digested with *Hind*III exhibited two bands at 7.1 and 4.7 kb, while the DNAs of YG7100 and TA1535 exhibited a single band whose molecular size was 4.7 and about 20 kb, respectively. From these results, we concluded that the *ada*$_{ST}$ gene of strain YG7101 was not replaced with the Km^r gene but that a single copy of the introduced DNA fragment carrying the Km^r gene was integrated at the 3' downstream side of the *ada*$_{ST}$ gene on the chromosome (Fig. 4, Type B). Since YG7102, the derivative of group B', exhibited an intense extra band at 7.8 kb along with bands at 8 and 13 kb (Fig. 2A), we suggest that multiple copies, rather than a single copy, of the introduced DNA fragment were integrated at the 3' downstream side of the *ada*$_{ST}$ gene of this strain (Fig. 4, Type B').

The digests of DNA of strain YG7103, the Km^r derivative of group C, exhibited a unique banding pattern. The DNA of strain YG7103 exhibited three bands with molecular sizes of 4.2, 4.0, and 1.2 kb when digested with *Eco*RV and exhibited two bands at about 20 and 4.7 kb when digested with *Hind*III. We suggest that strain YG7103 was not a true replacement either: a single copy of the Km^r gene was integrated at the 5' upstream side of the *ada*$_{ST}$ gene in this strain (Fig. 4, Type C).

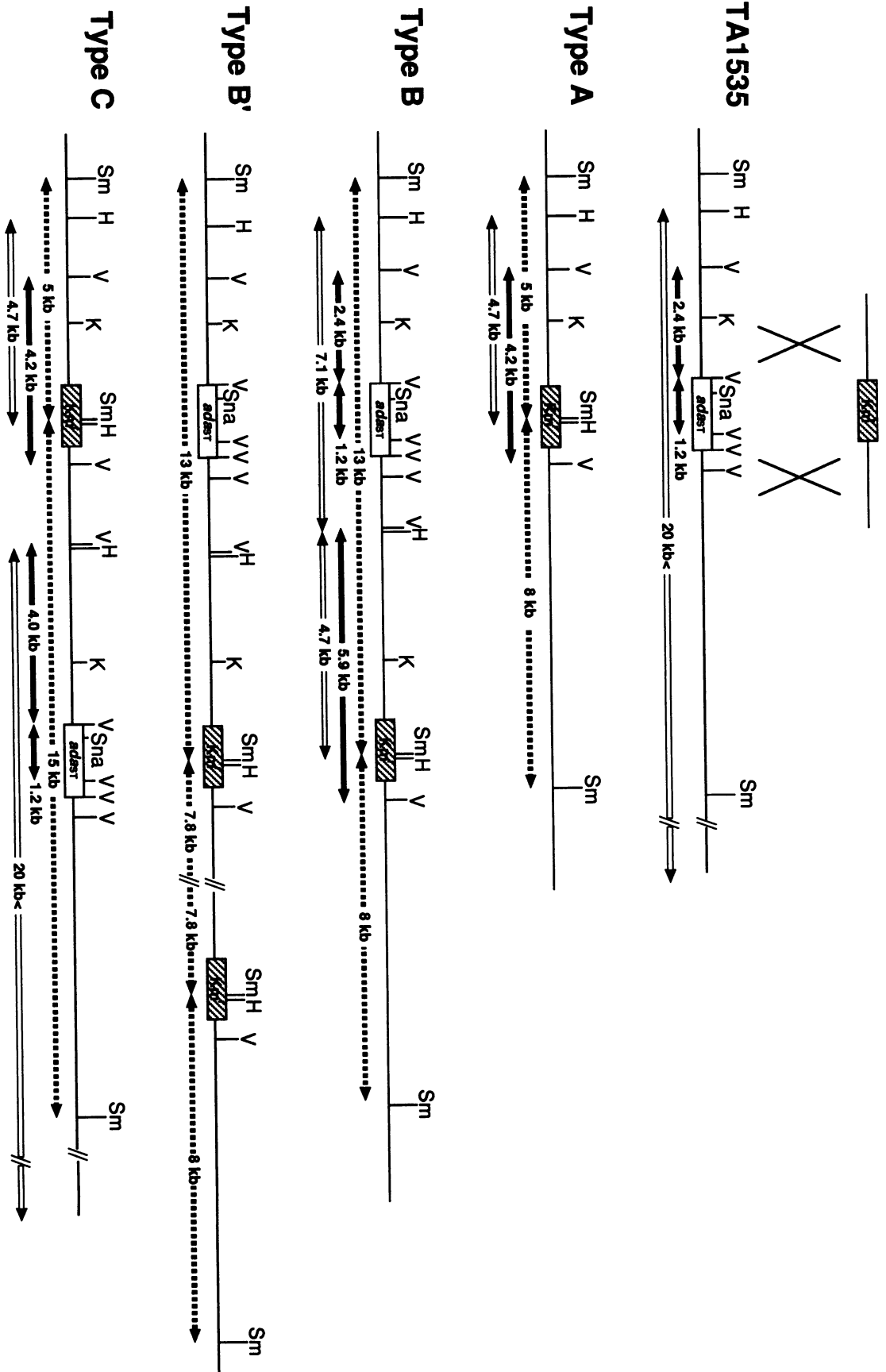Sensitivities of an *ada*$_{ST}$-deletion derivative of *S. typhimu-*

FIG. 4. Partial restriction map of the ada_{ST} gene and its surrounding chromosomal region in *S. typhimurium* TA1535 and its Km^r derivatives. Type A represents the structure of *S. typhimurium* YG7100, in which the ada_{ST} gene is replaced by the Km^r gene. Type B represents the structure of *S. typhimurium* YG7101, in which the Km^r gene is integrated into the 3' downstream side of the ada_{ST} gene. Type B' represents the structure of *S. typhimurium* YG7102, in which multiple copies of the Km^r gene are integrated into the 3' downstream side of the ada_{ST} gene. Type C represents the structure of *S. typhimurium* YG7103, in which the Km^r gene is integrated into the 5' upstream side of the ada_{ST} gene. The open box and the hatched box indicate the ada_{ST} gene and the Km^r gene, respectively. The positions and sizes of restriction DNA fragments shown in Fig. 2 and 3 are also indicated: dotted arrows, *Sma*I-*Sma*I DNA fragments that would hybridize to the probe DNA of the Km^r gene; solid arrows, *Eco*RV-*Eco*RV DNA fragments that would hybridize to the probe DNA of the *Kpn*I-*Sna*BI DNA fragment containing part of the ada_{ST} gene; open arrows, *Hin*dIII-*Hin*dIII DNA fragments that would hybridize to the probe DNA of the *Kpn*I-*Sna*BI DNA fragment containing part of the ada_{ST} gene. Restriction enzymes: Sm, *Sma*I; H, *Hin*dIII; V, *Eco*RV; K, *Kpn*I; Sna, *Sna*BI.
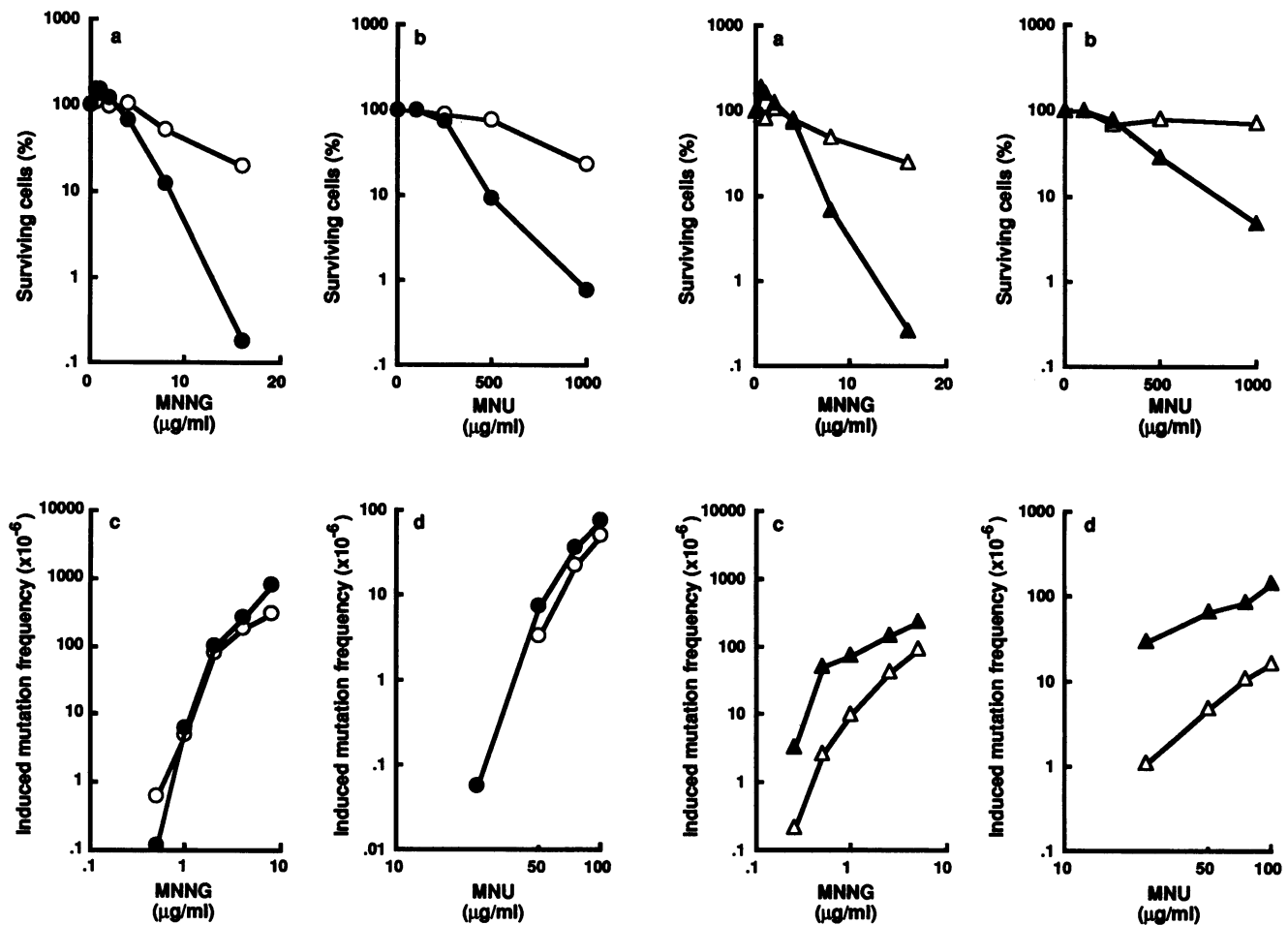
(A) S. typhimurium

(B) E. coli

FIG. 5. Effects of deletions of the ada_{ST} gene on the cell killing and mutagenesis induced by methylating agents in S. typhimurium (A). The results for E. coli strains with or without the ada gene are also presented for comparison (B). One milliliter of log-phase culture was treated with various concentrations of MNNG (a and c) or MNU (b and d) for 5 min at 37°C. Symbols: O, S. typhimurium TA1535; ●, S. typhimurium YG7100; △, E. coli AB1157; ▲, E. coli GW7101.

rium TA1535 to methylating agents. We compared the sensitivities of strain YG7100, an ada_{ST}-deletion derivative of S. typhimurium TA1535, to the cell-killing and mutagenic effects of MNNG and MNU with those of the parent strain TA1535 (Fig. 5A). Strain YG7100 exhibited much higher sensitivities to the cell-killing effects of MNNG and MNU than did the parent strain TA1535. However, the sensitivities of strain YG7100 to the mutagenic effects of MNNG and MNU were almost the same as those of the parent strain. In contrast to S. typhimurium, E. coli GW7101, an ada-deletion derivative (35), exhibited much higher sensitivities to the mutagenic effects as well as to the cell-killing effects of MNNG and MNU than did the parent strain AB1157 (Fig. 5B). The slopes of lines representing the induced-mutation frequencies of S. typhimurium in response to MNNG and MNU were steeper than those of lines representing the frequencies of E. coli, suggesting that S. typhimurium cells exhibit a threshold in their response to the mutagenic effects of MNNG and MNU. S. typhimurium YG7100 exhibited almost the same levels of sensitivities to the mutagenic and cell-killing effects of N-ethyl-N'-nitro-N-nitrosoguanidine

and N-ethyl-N-nitrosourea as did the parent strains TA1535 (data not shown). E. coli GW7101 also exhibited almost the same levels of sensitivities to the mutagenic and cell-killing effects of the ethylating agents as did the parent strain AB1157 (data not shown). These results suggested that neither Ada_{ST} of S. typhimurium nor Ada of E. coli efficiently removes an ethyl group from the DNA lesion.

DISCUSSION

We have developed a rapid and simple method for gene disruption in S. typhimurium. A feature of the method is treatment of the linear DNA fragments carrying a drug resistance gene flanked by chromosomal DNA on either side with T4 DNA ligase before they are introduced into S. typhimurium. This preligation method can be applied to any gene provided that cloned DNA is available and the location of the target gene within the cloned DNA fragment is known. One of the advantages of this method is that it can be carried out in a wild-type genetic background. Since it is not necessary to use a strain that is mutant in recD, recBC sbcB,
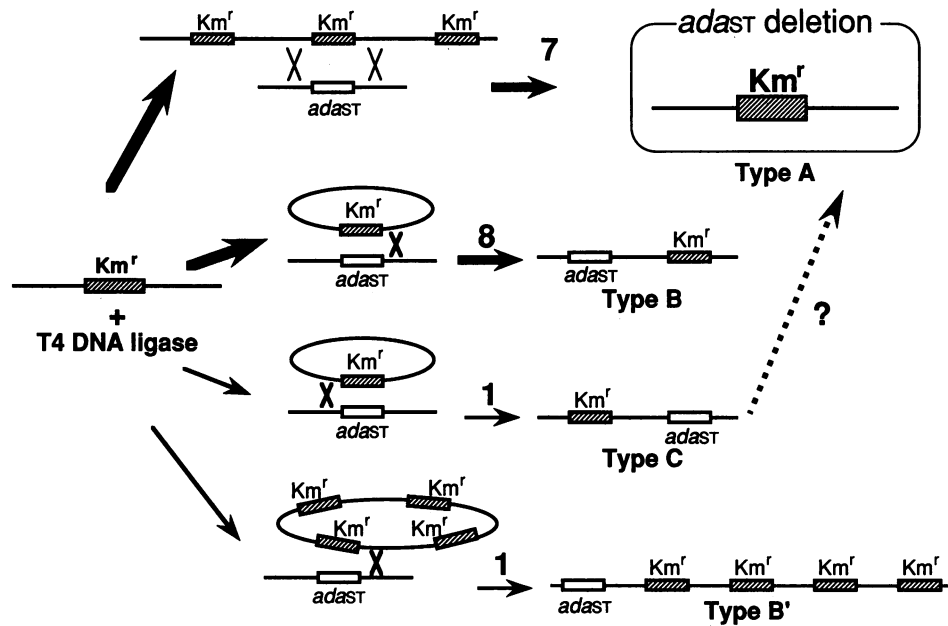
FIG. 6. Proposed mechanism by which the ada_{ST} gene on the chromosome of S. typhimurium is replaced with the Km^r gene. The replacement could occur by double crossovers on either side of the ada_{ST} gene. In this case, the linear multimer DNA molecules formed by the treatment with T4 DNA ligase could be involved. Of 17 Km^r derivatives of S. typhimurium TA1535, 7 were true replacements as shown in Fig. 2, 3, and 4. The remaining 10 Km^r derivatives were not true replacements but were cointegrates of the Km^r gene into the vicinity of the ada_{ST} gene. The formation of these cointegrates could be economically explained by suggesting that a single crossover occurred at one side of the ada_{ST} gene. Of the 10 cointegrates, structure types B and B' were found more frequently than was structure type C (eight B and one B' versus one C). This suggests that the crossover takes place at the 3' downstream side of the ada_{ST} gene more frequently than at the 5' upstream side. Alternatively, structure type C may be more prone to be segregated into the true replacement type A than is structure type B. The open and hatched boxes indicate the ada_{ST} gene and the Km^r gene, respectively.

or polA, this method is quite useful for carrying out gene replacement in enteric bacteria for which such mutants are unavailable. This method is, of course, applicable and useful for E. coli. In fact, we have disrupted a mutM gene encoding 8-hydroxyguanine DNA glycosylase of E. coli by using this method (26a). The use of a wild-type genetic background also prevents any problems associated with the use of mutant strains, e.g., the poor growth of a recBC sbcB strain of E. coli. The only requirement for the host strain is that it must be proficient in homologous recombination. Another advantage of this method is that it can be carried out by using conventional plasmids such as pBR322 or pBluescript. Since it is not necessary to use any special plasmids such as those having a temperature-sensitive pSC101 replicon (11) or those that can replicate only in Pseudomonas aeruginosa (6), it is not necessary to subclone the DNA region containing a target gene into the plasmids. The only requirement for the plasmid is that it must have restriction enzyme sites which are suitable for separation of the replication origin from the target gene.

The treatment of the linear DNA fragments with T4 DNA ligase substantially increased the number of Km^r colonies of strain TA1535 per transformation (Table 2). When the treated DNA was introduced into S. typhimurium by electrotransformation, the number of Km^r colonies was more than 100 even at 0.1 μg of DNA. Multimer formation mediated by T4 DNA ligase probably protects the linear DNA fragments from digestion by exonuclease and thus increases the chance of homologous recombination between the introduced DNA fragments and the chromosomal DNA of S. typhimurium. The treatment with T4 DNA ligase could produce both linear multimer DNA fragments and circular

(multimer) DNA molecules. As discussed below, it appears that both products are involved in the occurrence of Km^r colonies.

Of 17 Km^r derivatives of strain TA1535, about 40% (7 of 17) were true replacements, in which the ada_{ST} gene on the chromosome was exactly replaced with a single copy of the Km^r gene (Fig. 6). The formation of these cointegrates could be economically explained by suggesting that a single crossover occurred at one side of the ada_{ST} gene. If so, the linear multimer DNA molecules formed by the treatment with T4 DNA ligase could be involved in double crossovers. It is worth noting that there were no derivatives whose ada_{ST} gene was replaced with multiple copies of DNA fragments carrying the Km^r gene. The remaining 10 Km^r derivatives were not true replacements but were cointegrates of the Km^r gene into the vicinity of the ada_{ST} gene (Fig. 6). These 10 derivatives did not exhibit high sensitivities to the cell-killing effects of MNNG (Fig. 2). The formation of these cointegrates could be economically explained by suggesting that a single crossover occurred at one side of the ada_{ST} gene. If so, the circular monomer DNA molecules would be involved in the formation of such cointegrates. Interestingly, there appears to be a bias for the structure of the cointegrates: structure types B and B' (Fig. 4) were found more frequently than was structure type C (nine versus one). This suggests that the crossover takes place more frequently at the 3' downstream side of the ada_{ST} gene than at the 5' upstream side of the gene. Although the lengths of the chromosomal DNAs on the 3' and 5' sides of the Km^r gene on the linear DNA were not the same, this could not completely explain the bias for the site of crossover. Cointegrate types B and C may form the true replacement (type A) by deletion of the

ada_{ST} gene. Thus, the alternative explanation for the apparent bias is that the structure of cointegrate type C may be more prone to be segregated into the true replacement structure than is the structure of type B, while the crossover takes place almost equally at the 3' and 5' sides of the ada_{ST} gene. The exact reason for the preference for structure types B and B' over type C is not known. Structure type B', which was found in only 1 of 17 cases, could be formed by integration of multiple copies of the introduced DNA fragments carrying the Km^r gene at the 3' downstream side of the ada_{ST} gene. In this case, the circular multimer DNA molecules may be involved (Fig. 6).

In *E. coli*, the Ada protein methylated at the Cys-69 residue increases the expression of the *ada* gene itself as well as that of the *alkA* gene (19). The Ada protein repairs mutagenic lesions, i.e., O^6-methylguanine and O^4-methylthymine, whereas the product of *alkA* gene, 3-methylguanine-DNA glycosylase II, repairs lethal lesions, i.e., 3-methylpurine and 2-methylpyrimidine. Thus, it seems reasonable that strain GW7101, an *ada*-deletion mutant of *E. coli*, exhibited hypersensitivities to both the mutagenic and cell-killing effects of MNNG and MNU (Fig. 5B). In contrast, strain YG7100, an *ada*-deletion derivative of *S. typhimurium* TA1535, did not exhibit higher sensitivities to the mutagenic effects of MNNG and MNU than did the parent strain TA1535 (Fig. 5A). This suggests that the Ada_{ST} protein does not play a major role in protecting *S. typhimurium* against the mutagenic effects of the methylating agents.

In *E. coli*, there is a second MT, which is the predominant MT in unadapted cells (20, 29, 30, 35). The second MT, the Ogt protein, repairs O^6-methylguanine and O^4-methylthymine but not methylphosphotriesters (20). The amino acid sequence of this 19-kDa protein has homology with the C-terminal domain of the Ada protein (29). The expression of the *ogt* gene is constitutive (20). Rebeck et al. identified an active 19-kDa MT in unadapted *S. typhimurium* cells (31). Recently, we isolated an *S. typhimurium* gene which showed 77% similarity to the *ogt* gene of *E. coli* at the nucleotide level (48a). Thus, we suggest that the Ogt_{ST} protein but not the Ada_{ST} protein plays a major role in protecting cells against the mutagenic effects of methylating agents in *S. typhimurium*. An ogt_{ST}-deletion derivative of *S. typhimurium* TA1535 should be very sensitive to the mutagenic effects of alkylating agents.

Unlike the sensitivity to the mutagenic effects of methylating agents, strain YG7100, an ada_{ST}-deletion derivative of TA1535, exhibited hypersensitivities to the cell-killing effects of MNNG and MNU, similar to the response of the *ada* mutant of *E. coli* (Fig. 5). This suggests that the Ada_{ST} protein plays an important role in protecting the cells against the cell-killing effects of methylating agents. The ada_{ST} gene was initially isolated as a gene of *S. typhimurium* which can complement the lethal effects of MNNG on an *ada*-deletion derivative of *E. coli* (10). Thus, it seems likely that the Ada_{ST} protein can induce the expression of the *E. coli alkA* gene. Although the Ada protein of *E. coli* initially appeared to regulate the *ada* and *alkA* genes in a similar fashion, it has been proposed that different regulation mechanisms are involved in the induction of expression of *alkA* and *ada* of *E. coli* (37). The N-terminal half of the Ada protein is sufficient to induce activation of *alkA* transcription but is incapable of activation of *ada* transcription in vivo (37). The methylated N-terminal domain of Ada protein can promote *alkA* transcription, but this domain, methylated or unmethylated, cannot activate *ada* transcription in vitro (1, 49). The spacings between the Ada-binding site (Ada box) and the pro-

posed −35 site in *ada* and *alkA* of *E. coli* are different: the *ada* promoter contains the Ada box several base pairs away from the −35 region, whereas the *alkA* promoter contains an Ada box that almost completely overlaps the −35 region (7, 12, 26, 36). By analogy to the N-terminal domain of the *E. coli* Ada protein, we suggest that the Ada_{ST} protein has different abilities to function as a transcriptional activator for the ada_{ST} and *alkA* genes of *S. typhimurium*: the methylated Ada_{ST} protein may efficiently induce the expression of the *alkA* gene of *S. typhimurium* but not that of the ada_{ST} gene itself. This would preferentially increase resistance to the cell-killing effects of MNNG and MNU when the cells are exposed to the methylating agents.

ACKNOWLEDGMENTS

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