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A New Gene (*alkB*) of *Escherichia coli* That Controls Sensitivity to Methyl Methane Sulfonate

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Seven mutants of *Escherichia coli* were isolated that are sensitive to methyl methane sulfonate but not to UV light. They exhibited decreased host cell reactivation capacity for methyl methane sulfonate-treated phage λ. Five of the mutations were mapped in the same region as *alkA* (previously called *alk*) and may indeed be identical to known mutations. Another mutation was found near *nalA*, and the gene responsible was named *alkB*. Its phenotype was different from that of *ada*, since the *alkB* mutant exhibited a normal adaptive response to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. A third type of mutation was mapped near *polA*, but this mutant contained an almost normal level of DNA polymerase I activity.

Alkylating agents induce DNA damages which may cause either killing of cells or induction of mutation and cancer. Most of such damages are subjected to common cellular DNA repair mechanisms, such as excision repair and postreplication repair (see references 10 and 26 for reviews). It has been shown, moreover, that by pretreatment with a sublethal dose of alkylating agents, cells become resistant to mutagenic and killing effects of subsequent treatment with higher concentrations of the agents (2, 21). The latter process is called adaptive response.

To analyze the precise mechanisms for repair of alkylated DNA and to correlate the alkylation products with the biological consequences, it seemed to be necessary to isolate mutants that are specifically sensitive to alkylating agents. Two types of such mutants have been isolated, *alkA* (previously called *alk*) (27, 28) and *tagA* mutants (14). Recent studies have revealed that these genes control the formation of enzymes that catalyze the liberation of certain alkylated bases from DNA (7, 13, 25). In addition, *ada* and *adc* mutants have been isolated which are defective in controlling mechanisms to induce the adaptive response to alkylating agents (12, 23). In view of the complexity of the DNA repair mechanisms, there must be more genes that control these processes.

In this paper we report the isolation and characterization of a new mutant that is specifically sensitive to methyl methane sulfonate (MMS). The genetic locus responsible has been designated *alkB*.

MATERIALS AND METHODS

Bacteria. The *Escherichia coli* K-12 strains used in this study are listed in Table 1.

Chemicals. MMS and ethyl methane sulfonate (EMS) were purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan, and Eastman Kodak Co., New York, respectively. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was obtained from Yoneyama Yaku-hin Kogyo Co., Osaka, Japan.

Media. M9S buffer without a carbon source was prepared as described previously (27). M9S buffer supplemented with 0.4% glucose, 5 μg of thiamine per ml, and 20 μg of each required amino acid per ml was used as supplemented M9 medium. LB medium and E medium were prepared as described previously (4). Nutrient broth contained 10 g of polypeptone, 5 g of extract of bonito meat, and 3 g of NaCl per liter (pH 7.2).

Isolation of mutants. Exponentially growing cells were harvested and treated with 50 μg of MNNG per ml in 0.1 M citrate buffer at pH 5.5 and 37°C for 35 min (50% killing), or with 1.5% EMS in M9S buffer containing 0.2 M Tris-hydrochloride at pH 7.5 and 37°C for 120 to 160 min (30 to 70% killing). The cells were diluted with LB medium and incubated overnight at 37°C. After appropriate dilution, the cells were plated on LB medium plates, and the colonies formed were replica plated on LB medium plates containing 0.05% MMS. After incubation at 37°C overnight, MMS-sensitive colonies were selected from the master plates and purified, and their MMS sensitivity was checked by the rapid test (27). About 0.8% of the colonies examined were MMS sensitive. All MMS-sensitive colonies were further tested for their resistance to UV light (a single dose of 40 J/m^2), and cells which were as resistant to UV light as the parental strain were selected. Among 179 colonies examined, 37 colonies were MMS sensitive and UV resistant. Finally, the host cell reactivation capacity for MMS-treated phage λ was examined, and mutants which showed reduced host cell reactivation capacity were selected.

Sensitivity to MMS. The rapid test for determining the MMS sensitivity of cells was performed as described by Yamamoto et al. (27). The gradient plate method was also used to determine the MMS sensitiv-

TABLE 1. Bacterial strains used

Strain	Genotype or phenotype	Source or derivation
AB1157	*thr-1 leu-6 proA2 his-4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 strA31 supE44*	Howard-Flanders (11)
YY41	As AB1157, but *recA1*	Yamamoto (28)
MS23	As AB1157, but his^+ *alkA1*	Yamamoto (28)
PJ5	As AB1157, but *ada-5*	Jeggo (12)
HK2	As AB1157, but *alk-2*	MNNG treatment of AB1157
HK12	As AB1157, but *alk-12*	EMS treatment of AB1157
HK22	As AB1157, but *alkB22*	EMS treatment of AB1157
HK26	As AB1157, but *alk-26*	EMS treatment of AB1157
HK29	As AB1157, but *alk-29*	EMS treatment of AB1157
HK32	As AB1157, but *alk-32*	EMS treatment of AB1157
HK34	As AB1157, but *alk-34*	EMS treatment of AB1157
HK70	As HK22, but *nalA*	Spontaneous Nal^r mutant of HK22
HK80	As AB1157, but *nalA*	P1(HK70) × AB1157; Nal^r transductant
HK82	As AB1157, but *nalA alkB22*	P1(HK70) × AB1157; Nal^r MMS^s transductant
JG138	*rha thy lacZ polA1*	Richardson (3)
JG139	As JG138, but $polA^+$	Richardson (3)
W3623	*trp gal str*	Shimada
AB2277	*ilvE12 metE46 his-4 trp-3 pro-2 thi-1 mtl-1 mal-1 ara-9 gal-2 lac-1* or *lac-4* $T6^r$ *str*	Adelberg (5)
CSH56	*ara* Δ(*lac pro*) *supD nalA thi*	Cold Spring Harbor (19)
KK446	*thi-1 leuB6 thyA6 thi-1 lacY1 rpsL67 tonA21* λ^- *supE44 deoC1 deoB37 nrdB2*	Bachmann (8)
PK191	Hfr Δ(*proB-lac*) *thi-1 relA1*? λ^- *supE44*; injection order: *supD his*	Bachmann (18)
BE5408	Hfr prototroph; injection order: *his supD trp*	Yamamoto (27)
KL98	Hfr prototroph; injection order: *dsdA aroC*	Low (18)
KL16	Hfr *thi-1 relA1* λ^-; injection order: *lysA thyA*	Bachmann (18)
KL14	Hfr *thi-1 relA1* λ^-; injection order: *argG gltB argR*	Bachmann (18)
KL25	Hfr *supE42* λ^-; injection order: *ilvE metE argE*	Bachmann (18)
Ra-2	Hfr *sfa-4 supE42 mal-28* λ^r λ^-; injection order: *metB argE*	Bachmann (18)
KL209	Hfr *thi-1 malB16 supE44* λ^r λ^-; injection order: *argE metB*	Bachmann (18)

ity of mutants semiquantitatively (24). Square-built LB agar plates (9.5 by 13.5 by 0.8 cm) with a gradient of MMS concentration (0 to 0.05% or 0 to 0.1%) were used. A freshly prepared overnight culture was 10^{-2} diluted, and a loopful of it was streaked from the high to the low concentration of the MMS gradient on the plate. After overnight incubation at 37°C, the degree of confluent growth on the gradient was measured. A 1% solution of MMS is equivalent to 118 mM.

Genetic mapping. At first, mutations were roughly mapped on the *E. coli* genetic map (1) by the replica plating method of Low (18). Mutants were conjugated with some Hfr strains with different points of origin and different orientations on M9 selective plates. The selective markers used were *thr*, *leu*, *proA*, *argE*, and *his*. The counterselection was done with 100 μg of streptomycin per ml. The resulting recombinants were tested for MMS sensitivity by replicating on LB agar plates containing 6 mM (0.05%) MMS, to estimate the relative position of the mutation and the point of origin.

Mutations were more precisely mapped by conjugation and P1 transduction as described by Miller (19). Supplemented E medium agar plates were used for selection of P1 transductants. $nrdB^+$ transductants were selected with resistance to 1 mg of hydroxyurea per ml. For transduction of *nalA*, P1-infected cells were washed twice with E medium, incubated for 2 h at 37°C in LB medium containing 5 mM sodium citrate for expression of the recessive Nal^r phenotype, and selected on LB agar plates containing 40 μg of nalidixic acid per ml. Due to the relatively high frequency of spontaneous Nal^r mutations, the cotransduction frequency with *nalA* might be underestimated.

Enzyme activity. Cell-free crude extracts were prepared as described previously (27). DNA polymerase I activity was assayed as described previously (6).

RESULTS

Isolation of MMS-sensitive mutants. Strain AB1157 was mutagenized by MNNG or EMS, and mutants that exhibited increased sensitivity to MMS but not to UV light were selected. We isolated seven mutants (Table 2), one (HK2) from an MNNG-treated culture and the others

TABLE 2. Characteristics of mutants isolated

Strain	Mutation	Sensitivity to:		HCR capacity for MMS-treated phage λ[a]	
		MMS[b]	UV light[c]	Survival	Ratio of survival to that of wild type
Reference strains					
JG139	Wild type	1.0	R	1.5×10^{-2}	1.0
JG138	*polA1*	<0.01	S	$<1.0 \times 10^{-7}$	$<10^{-5}$
YY41	*recA1*	<0.01	S	3.5×10^{-2}	1.5
MS23	*alkA1*	0.10	R	3.9×10^{-3}	0.17
AB1157	Wild type	1.0	R	2.3×10^{-2}	1.0
Mutants isolated					
HK2	*alk-2*	0.19	R	$<2.0 \times 10^{-3}$	<0.10
HK12	*alk-12*	<0.01	R	1.6×10^{-3}	0.070
HK26	*alk-26*	0.12	R	1.3×10^{-3}	0.057
HK32	*alk-32*	0.09	R	9.3×10^{-4}	0.040
HK34	*alk-34*	0.38	R	2.5×10^{-3}	0.11
HK22	*alkB22*	0.13	R	1.5×10^{-3}	0.065
HK29	*alk-29*	0.64	R	2.9×10^{-3}	0.13

[a] Host cell reactivation (HCR) capacity is expressed as the surviving fraction of λ*vir* exposed to 50 mM MMS for 80 min at 37°C.

[b] MMS sensitivity was determined on gradient plates containing 0 to 0.05% or 0 to 0.1% MMS. The length of the confluent growth zone of each mutant strain was divided by that of its parental strain.

[c] UV sensitivity was examined by streaking cultures on LB medium plates and irradiating at 40 J/m^2. R, Resistant; S, sensitive.

from EMS-treated cultures. Mutant HK12 was the most sensitive to MMS, and the other mutants exhibited a sensitivity intermediate between those of mutant HK12 and the wild-type strain.

The mutants were less capable of reactivating MMS-treated phages λ than was the parental strain, AB1157. These results strongly suggest that the increased sensitivity of the mutants to MMS is not caused by enhanced permeability but may be due to a defect in the repair of MMS-induced damages in their own or phage DNA. Since these properties of the mutants are similar to those of the *alkA1* mutant previously isolated (27), these mutations may be collectively called *alk*.

Location of the mutations on the genetic map. (i) Conjugation. By the replica plating method, all of the mutations except *alk-29* were found to be located in the region between the origins of strains PK191 and KL16. *alk-29* appeared to be in the region between the origins of strains KL14 and Ra-2. The origins and the directions of transfer of the Hfr strains used and relevant genetic markers are indicated in Fig. 1. More precise mapping was performed by crossing each mutant with some Hfr strains (Table 3). A number of His$^+$ Str^r recombinants received the donor MMS^r character when strains HK2, HK12, HK26, HK32, and HK34 were crossed with strain PK191 or BE5408. This indicates that the mutations of these strains reside in the region where the *alkA1* gene is located (43 to 45 min).

The cross of strain HK22 with strain BE5408 yielded no His$^+$ MMS^r recombinants, whereas a number of His$^+$ MMS^r recombinants were produced in the cross of HK22 with PK191, KL98, or KL16. Therefore, *alkB22* is located between 45 and 51 min on the linkage map.

The MMS^r character was readily transfered to strain HK29 from strains KL14, KL25, and KL209, but not from strain Ra-2. This indicates that the *alk-29* mutation is located between the origins for strains KL25 and Ra-2 (84 to 88 min).

The different chromosomal locations of *alkA1*, *alkB22* and *alk-29* suggest that they are mutations in different genes, although their phenotypes are similar.

(ii) P1 transduction. A P1 lysate prepared from strain W3623 (*his*$^+$) was applied to MMS-sensitive strains, and the MMS sensitivity of His$^+$ transductants was examined. We found that *alk-12*, *alk-26*, *alk-32*, and *alk-34* were 7 to 27% cotransducible with *his*. Data with *alk-2* were not available because strain HK2 was unable to grow on supplemented E medium agar plates.

Consistent with the data from Hfr crosses, no His$^+$ MMS^r recombinant appeared in a similar transduction experiment with strain HK22

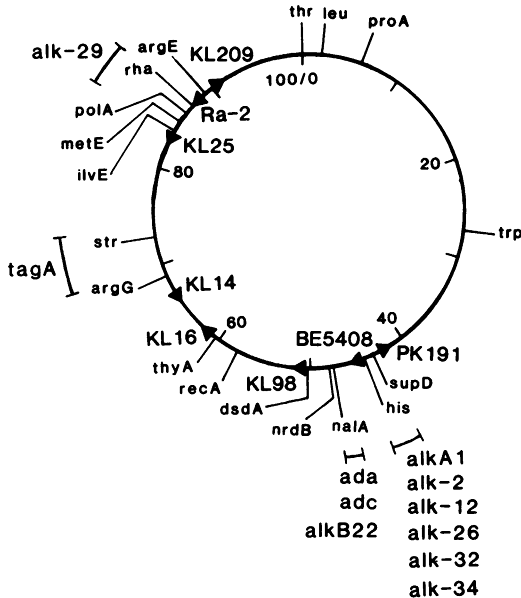

FIG. 1. Genetic map of *E. coli* K-12 showing the locations of genes responsible for MMS sensitivity, some relevant markers, and the origins and directions of transfer of the chromosome of Hfr strains.

(*alkB22*). The precise map position of *alkB22* was then determined by using *nalA* and *nrdB* as markers. First, a P1 lysate prepared from the nalidixic acid-resistant strain CSH56 (*nalA alk*$^+$) was applied to strain HK22 (*nalA*$^+$ *alkB22*), and Nal^r transductants were selected. Of 203 transductants examined, 45 exhibited the donor MMS^r character, indicating that *alkB22* is 22% cotransducible with *nalA*. In a second transduction experiment, strain KK446 (*nrdB*), which is sensitive to hydroxyurea, was infected with P1 grown in strain HK82 (*nalA alkB22*), and hydroxyurea-resistant transductants were selected. Of 130 NrdB$^+$ transductants, 32 were MMS^s and 73 were Nal^r. Only three colonies were MMS^s Nal^s. These results indicate that the relative gene order between 47 and 48 min is *alkB22-nalA-nrdB*.

To determine the approximate map position of *alk-29*, a P1 lysate prepared from strain HK29 was applied to strains JG139 (*rha*) and AB2277 (*metE ilv*). *alk-29* was cotransducible with *metE* and *rha* at frequencies of 4 and 9%, respectively, but was not cotransducible with *ilv*. Thus, the *alk-29* mutation is located between 85 and 87 min on the chromosome. In this region of the chromosome there is the *polA* gene, which encodes DNA polymerase I (9, 15). Although *polA* mutants are sensitive to UV light, the *alk-29* mutant was only slightly sensitive to UV light. Moreover, the *alk-29* mutant possessed an almost normal level of DNA polymerase I activity (data not shown).

Characterization of *alkB* mutation. A spontaneous Nal^r mutant, HK70, was isolated from mutant HK22, and *alkB22* was transduced to strain AB1157 with nalidixic acid resistance as selective marker to yield a Nal^r MMS^s strain, HK82. In the same transduction experiment a Nal^r MMS^r strain, HK80, also was isolated. Strains HK82 (*nalA alkB22*) and HK80 (*nalA alkB*$^+$) with the same AB1157 background were used to determine the effect of the *alkB22* mutation.

(i) Cell growth. The growth rate of the original strain, HK22, was slightly lower than that of the parental strain, AB1157. However, strains HK82 and HK80 did not show a significant difference in the growth rate under ordinary growth conditions. Thus, the mutation does not seem to affect the growth rate. Spontaneous mutation frequencies of strains HK82 and HK80 were almost the same when determined by measuring the mutation to rifampicin resistance.

(ii) Host cell reactivation. Strain HK82 was

TABLE 3. Crosses of mutants with various Hfr strains[a]

MMS^s mutant strain	MMS^r Hfr strain	No. of recombinants		Frequency of MMS^r recombinants (%)
		Total	MMS^r	
		His$^+$ Str^r	His$^+$ Str^r MMS^r	
HK2	PK191	102	89	87
	BE5408	79	45	57
HK12	PK191	89	58	65
	BE5408	102	39	38
HK26	PK191	105	76	72
	BE5408	102	56	55
HK32	PK191	87	10	11
	BE5408	31	20	65
HK34	PK191	100	70	70
	BE5408	89	58	65
HK22	PK191	115	40	35
	BE5408	103	0	0
	KL16	100	61	61
	KL98	105	52	50
		Ilv$^+$ Str^r	Ilv$^+$ Str^r MMS^r	
HK29	KL14	62	31	50
	KL25	67	28	42
	Ra-2	72	0	0
	KL209	48	16	33

[a] Mutants were crossed with Hfr strains for 30 min at 37°C, and His$^+$ Str^r or Ilv$^+$ Str^r recombinants were selected. The MMS sensitivity of each recombinant was then determined by the rapid test.

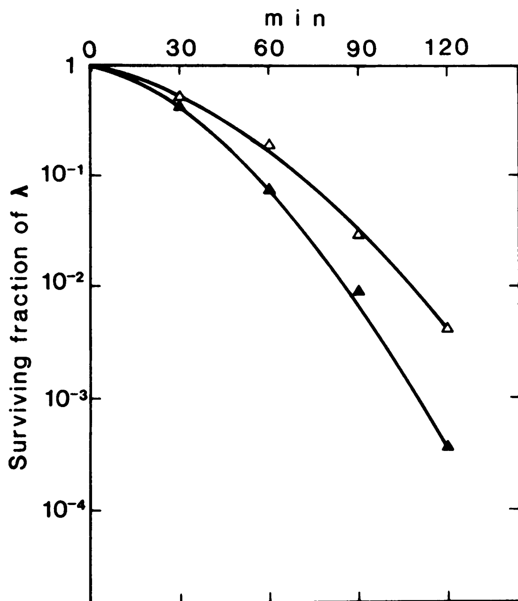

FIG. 2. Host cell reactivation capacity for MMS-treated phage λ. Phage λ*papa* was incubated with 50 mM MMS in M9S buffer for various times at 37°C and plated with a fresh overnight culture of cells suspended in 10 mM $MgSO_4$ on nutrient broth agar plates. Symbols: △, strain HK80 (*alkB*$^+$); ▲, strain HK82 (*alkB22*).

less capable of reactivating MMS-treated phage λ than was strain HK80 (Fig. 2), although the difference between HK82 and HK80 was not as great as that observed with the original *alkB22* strain, HK22, and its parent, AB1157 (Table 2). The UV sensitivity level of strain HK82 was essentially the same as that of strain HK80 (data not shown).

(iii) Adaptive response. As described above, *alkB22* is closely linked with *nalA*. In this region of the *E. coli* chromosome, the *ada* and *adc* mutations affecting the adaptive response to alkylating agents have been located (22). It has been shown that the *ada-5* mutant is sensitive to MNNG and produces MNNG-induced mutations at a high frequency. Thus, the yields of mutations in MNNG-treated cultures of *alkB22* and *ada-5* strains were compared. The normal adaptive response was induced in the *alkB22* mutant, and the occurrence of MNNG-induced revertant mutations in strains HK82 and HK80 was equally suppressed when the cells had been grown in a medium with a low concentration (0.1 μg/ml) of MNNG (Fig. 3B).

The adaptive response to the killing effect was next examined (Fig. 3A). The level of resistance to MNNG of *alkB22* cells preexposed to a low dose of MNNG was higher than that of the same cells without pretreatment, and the extent of the reactivation of *alkB22* cells was essentially the same as that of *alkB*$^+$ cells. From these results it is evident that a normal adaptive response takes place in the *alkB22* mutant.

Different actions of MMS and MNNG. The strains carrying mutations *alkB22* and *ada-5* exhibited completely different responses to two simple alkylating agents, MMS and MNNG.

(i) Sensitivity to alkylating agents. The *alkB22* mutant was very sensitive to MMS, whereas the *ada-5* mutant was as resistant to the chemical as were wild-type strains (Fig. 4A). On the other hand, the *alkB22* mutant exhibited only a slightly increased sensitivity to MNNG, whereas the *ada-5* mutant was the most sensitive to MNNG (Fig. 4B).

(ii) Induction of mutations. Specific responses of the *alkB22* and *ada-5* mutants to MNNG and

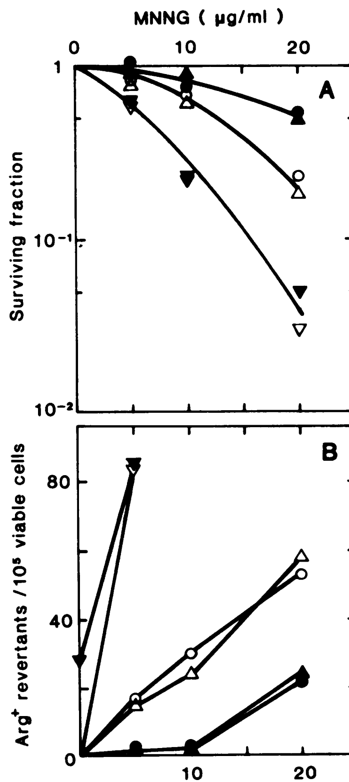

FIG. 3. Adaptive response of cells. Bacteria were exposed to various concentrations of MNNG for 5 min after growth with (closed symbols) or without (open symbols) 0.1 μg of MNNG per ml in supplemented M9 medium (pH 6) for 2 h at 37°C. (A) Survival of *E. coli* strains exposed to various concentrations of MNNG. (B) Reversion frequency (Arg^- [*argE*] → Arg^+) induced by MNNG. Symbols: △ and ▲, strain HK82 (*alkB22*); ○ and ●, strain HK80 (*alkB*$^+$); ▽ and ▼, strain PJ5 (*ada-5*).

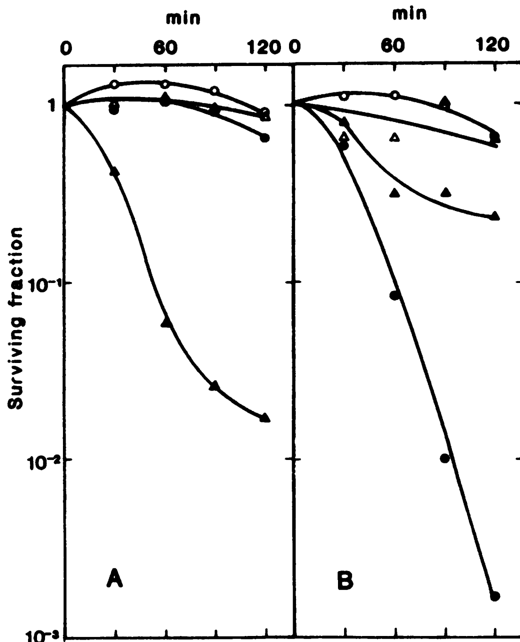

FIG. 4. Survival of *E. coli* strains exposed to MMS or MNNG. Cells were shaken with 6 mM (0.05%) MMS in supplemented M9 medium (pH 7) (A) or with 2 μg of MNNG per ml in supplemented M9 medium (pH 6) (B) for various times at 37°C. Symbols: ○, strain AB1157 (*ada*$^+$); ●, strain PJ5 (*ada-5*); △, strain HK80 (*alkB*$^+$); ▲, strain HK82 (*alkB22*).

MMS were observed when the frequencies of mutations induced by the two chemicals were compared. On treatment with MNNG, a large number of Arg$^+$ revertants appeared in the *ada-5* mutant but not in the *alkB22* mutant (Fig. 5B). On the other hand, MMS induced a number of revertants in the *alkB22* strain but not in the *ada-5* strain, although the frequency of mutations induced by MMS in the *alkB22* mutant was considerably lower than that induced by MNNG in the *ada-5* mutant (Fig. 5A).

DISCUSSION

Seven mutants which showed increased sensitivity to MMS but not to UV light were isolated in the present study. Since the mutants were unable to reactivate λ phage preexposed to MMS, it was suggested that they are defective in some step(s) for repair specific for alkylated DNA. According to the mapping data, these mutants were classified into three groups: group A mutants, whose mutations are located at 43 to 45 min on the linkage map, group B mutant, at 47 to 48 min, and group C mutant, at 85 to 87 min.

Two genes *tagA* and *alkA*, which cause similar phenotypes have been found in *E. coli* (14, 27). The *tagA* gene has been mapped at 70 to 74 min on the chromosome (7), and it has been shown that this gene controls a constitutive enzyme, 3-methyladenine-DNA glycosylase I, that releases 3-methyladenine from alkylated DNA (13, 14, 25). The *alkA* gene has been mapped at approximately 45 min, and recent biochemical studies have pointed out that it controls an inducible enzyme, 3-methyladenine-DNA glycosylase II, which catalyzes the liberation of 3-methyladenine, 3-methylguanine, and 7-methylguanine from the DNA (7, 13). The close chromosomal location and similar phenotypes of the *alkA* and group A mutants suggest that these mutations are alleles of a single gene, *alkA*. Recently, the *alkA* gene has been cloned (Y. Yamamoto, unpublished data), and the relationship of these mutations would be clarified by complementation tests with the cloned gene. In the region where *alkB22* was located, *ada* and *adc*, which are required for induction of the adaptive response to alkylating agents, have been mapped (12, 22, 23). The possibility that *alkB22* was an allele of the *ada* or *adc* gene was excluded because the mutant normally induced

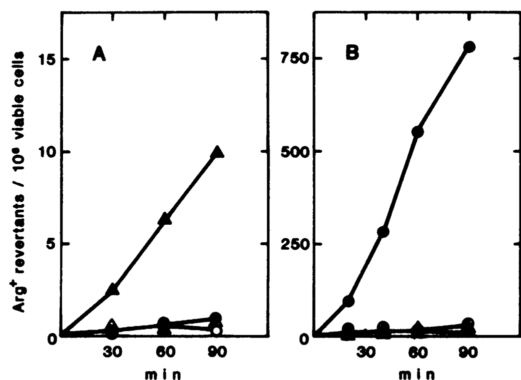

FIG. 5. Reversion frequency (Arg$^-$ [*argE*] → Arg$^+$) of *E. coli* strains induced by MMS or MNNG. (A) Bacteria were shaken with 1.2 mM (0.01%) MMS in supplemented M9 medium (pH 7) for various times at 37°C. At the times indicated, samples of the cultures were withdrawn, washed, and suspended in M9S buffer. After appropriate dilution, samples of the culture were plated on supplemented M9 agar plates without arginine with 2 ml of enriched M9 top agar containing 0.5 mg of arginine per ml for measuring the number of viable cells, and on the same plate with 2 ml of semienriched M9 top agar containing 1 μg of arginine per ml for measuring the number of Arg$^+$ revertant cells. The plates were incubated for 2 to 3 days at 37°C. (B) Bacteria were shaken with 0.2 μg of MNNG per ml in supplemented M9 medium (pH 6) for various times at 37°C. Symbols: ○, strain AB1157 (*ada*$^+$); ●, strain PJ5 (*ada-5*); △, strain HK80 (*alkB*$^+$); ▲, strain HK82 (*alkB22*).

both mutagenic and killing adaptation to MNNG treatment. Thus, *alkB22* seems to reside in a new gene that controls sensitivity to MMS. We propose that this gene be designated *alkB* and that the original *alk* gene, linked to *his*, be named *alkA*.

Of interest is the finding that *alkB22* and *ada-5* mutants respond to MMS and MNNG in quite different manners. Although both MNNG and MMS are simple alkylating agents, the amounts of methylated products produced in DNA by the two chemicals are different. O^6-methylguanine, a major premutational lesion in the DNA, is more abundantly produced by MNNG than by MMS (16, 17). It has been shown that a methyltransferase that transfers the methyl group from O^6-methylguanine in DNA to the protein itself is induced under the control of the *ada* gene (7, 12, 20). This explains, at least in part, why the *ada* mutant is particularly sensitive to MNNG and yields a high frequency of mutations when exposed to MNNG. It can be supposed that the *alkB* gene product may be involved in the repair of an alkylated base(s) that is produced specifically by MMS. It would be of interest to compare the rates of release or disappearance of various alkylated bases from DNA in *alkB* and $alkB^+$ strains.

Finally, *alk-29* may be a new type of mutation that has not been described before. The mutation has been located near *polA* on the chromosome, but the mutant exhibited almost normal sensitivity to UV light and possessed an almost normal level of DNA polymerase I.

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