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FORWARD MUTATION ASSAY FOR SCREENING CARCINOGENS BY ALKALINE PHOSPHATASE CONSTITUTIVE MUTATIONS IN *ESCHERICHIA COLI* K-12

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SUMMARY A new forward mutation assay was developed with *Escherichia coli* using alkaline phosphatase (APase) constitutive mutations as a genetic marker. Mutation in any one of the three regulator genes (*phoR*, *T* and *S*) is known to make the cell constitutive for APase synthesis and enable the mutants to form larger colonies on β-glycerophosphate plate under the condition of excess inorganic phosphate. This property was used for qualitative and quantitative assay of chemical mutagens.

Attempts were made to construct suitable strains for this assay by introduction of various genetic traits that might increase the sensitivity of mutation. Three known chemical mutagens (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl methanesulfonate (MMS), and 4-nitroquinoline-1-oxide (NQNO)) were employed as reference compounds in the quantitative assay. Among the strains constructed, a tester strain with genetic markers *tif-1*, *uvrA* and pKM101 was the most sensitive to these compounds, judging from tests on concentration-dependent mutagenic activity.

The merits and limitations of the present system are discussed.

INTRODUCTION

Since there is accumulating evidence of a close correlation between environmental agents with mutagenic activity and cancer, it is of considerable importance to develop a screening system for rapid and efficient detection of mutagenic agents. At present the assay system developed by Ames and his associates utilizing *Salmonella typhimurium* is the most widely used short-term microbial assay system (Ames et al., 1973). In this assay, specific lesions in the histidine operon are back-mutated or suppressed to allow the bacterium to synthesize histidine. Therefore, in this system several strains should be used simultaneously to provide a variety of genetic lesions as targets, because certain chemical classes show specificity with regard to the kind of mutations they induce and the se-

quences of DNA they attack.

An alternative to reversion assay is forward mutation assay, which can detect mutations that occur over a larger part of the DNA and any kind of genetic lesion that alters a particular phenotype. This system might thus detect some mutagens that are not detectable by reversion assay. Attempts to develop quantitative forward mutation systems with bacteria have been described recently (Ames et al., 1971; Mohn, 1973; Pueyo, 1978; Skopek et al., 1978a).

We developed a forward mutation assay with *Escherichia coli* K-12 which is based on the ability of alkaline phosphatase (APase) constitutive mutants to form large colonies on a selective plate. Synthesis of APase by the standard strain of *E. coli* K-12 is repressed when the cells are grown in medium containing excess inorganic phosphate, and the enzyme appears only after the medium has become depleted of inorganic phosphate (Torriani and Rothman, 1961). However, cells with mutation of the regulator genes *phoR*, *phoS*, and *phoT* produce the enzyme constitutively (Willsky et al., 1973), and this enables these mutant cells to form larger colonies with β-glycerophosphate as carbon source in the presence of excess phosphate on the selective plate (Torriani and Rothman, 1961).

The *E. coli* strains used for our assay were constructed by combining various genetic traits which were expected to increase the sensitivity of the mutation assay. These were *uvrA*, *tif-1*, and plasmid pKM101. This paper describes the assay system and results of qualitative and quantitative assays using some typical chemical mutagens.

METHODS AND RESULTS

1. *Chemicals*

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine(MNNG), 4-nitroquinoline-1-oxide(NQNO) and *N*-nitrosomethylurea were purchased from Nakarai Chemicals, Ltd; methyl methanesulfonate(MMS) was from Aldrich Chemical Co.; ethylene dibromide was from Wako Pure Chemical Industries, Ltd.; 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt (XP) and β-glycerophosphate (β-Gp) were from Sigma Chemical Co.

2. *Assay conditions*

1) Qualitative assay (Spot test)

Overnight liquid cultures for mutation assay were made in TG-C medium (consisting of 0.1 *M* Tris buffer (pH 7.2), 0.08 *M* NaCl, 0.02 *M* KCl, 0.02 *M* NH_4Cl, 1 m*M* $MgCl_2$, 2.5 m*M* Na_2SO_4, 0.2 m*M* $CaCl_2$, 2 μ*M* $FeCl_3$, 2 μ*M* $ZnCl_2$, 1% glucose, 0.64 m*M* KH_2PO_4, 0.2% casamino acids and 5 μg/ml thiamine) at 37°C. Then 0.1 ml of the overnight culture was spread on a TGp-C-XP plate (containing of 40 μg/ml XP, 1.5% agar and all the components TG-C medium except glucose, which was replaced by 0.33% β-Gp as the carbon source). Filter paper discs were placed on the plate, and test chemicals at appropriate concentrations in a volume of 20 μl were applied to these discs. The plate was then incubated for 3 days at 37°C for *tif*$^+$ strains and at 39°C for *tif-1* strains. APase constitutive mutant colonies were detected as larger colonies around the discs containing mutagen; the colonies appeared blue since the XP included in the plate stained the enzyme APase.

2) Quantitative assay

A 0.1 ml portion of the overnight culture and the test compound in 20 μl of dimethyl sulfoxide were added to 2 ml of top agar (0.6% NaCl and 0.5% agar) and the mixture was layered over a TGp-C-XP plate. Surviving cells were counted on a TGly-C plate (1.5% agar in TG-C medium with glycerol instead of glucose) with and without the test chemical. When *tif*$^+$ strains were used the plates were incubated for 3 days at 37°C. However, *tif-1* strains were used and the plates were incubated at 41°C or 37°C throughout, the sensitivity of mutagenicity was unexpectedly low. We, therefore, examined the optimal incubation conditions for the expression of *tif* dependent error prone repair function in the present assay by changing the length of the initial incubation periods at 41°C. As shown in Fig. 1, incubation of plates for 24 hr at 41°C and for a further 48 hr at 37°C gave the highest sensitivity to MMS, as examined by production of APase constitutive mutants. Therefore, we employed these incubation conditions when *tif-1* strains were used for the assay.

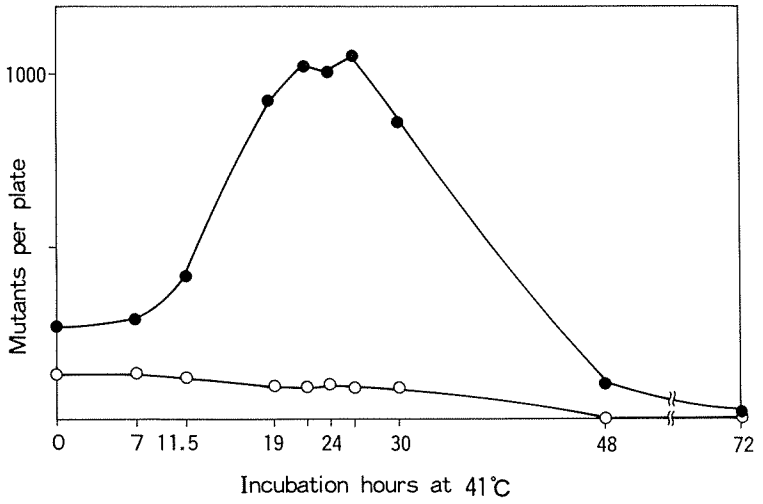


FIGURE 1. Determination of optimal incubation conditions for assay with *tif* strain (KI201). Numbers of APase constitutive mutant colonies formed on a TGp-C-XP plate (ordinate) with MMS (2 μl/plate, ●—●) or without MMS (○—○) are plotted against the initial incubation period at 41°C. The plates were incubated at 37°C for the rest of the total incubation period of 72 hr.

3. *Construction of the tester strains*

While we were trying to improve the sensitivity of the present assay system by constructing the tester strains with various genetic backgrounds, we noticed that some strains did not produce APase constitutive mutant colonies on TGp-C-XP plates with the mutagen (MNNG). Since one Hfr strain W2252 gave a positive response in the present assay, the responsive genetic character (named APM^+) was transferred from this strain by conjugation to APM^- strains (JM1 and GC714) for use in the assay.

uvrA, which is defective in excision repair of damaged DNA, is known to increase the sensitivity of assays (Kondo et al., 1970), and *tif-1*, which expresses error prone repair at high temperature, has been shown to enhance the mutagenicity by MMS (Walker, 1977). *sfiA* suppresses deletereous filamentation of the cell by *tif-1* expression at high temperature, but allows error prone repair function (George et al., 1975). We included strains with these genetic traits in our assay system.

Plasmid pKM101, which enhances the sensitivity to many mutagens (McCann et al., 1975a; Skopek et al., 1978b), was introduced into the tester strains by conjugation. The tester strains thus constructed are shown in Table 1.

4. *Application of the forward mutation assay system*

A qualitative spot test is a simple procedure for use in screening a large number of chemicals. The typical appearance of a positive result in this test using MNNG as mutagen is shown in Fig. 2. A circle of large blue colonies (APase constitutive mutants) is seen around the spot of the mutagenic chemical, which forms a concentration gradient from the point of application on incubation for 3 days.

TABLE 1. *Bacterial strains used*

Strain	Relevant genotype	Derivation or sources
JM1	wild	Castellazzi, et al. (1972)
KI101	APM^{+a}	Recombinant obtained from a cross: W2252 × JM1
KI111	APM^+, pKM101	KI101 into which pKM101 was introduced
GC714	*tif*-1, *sfiA*-11, *uvrA*	G. George
KI201	APM^+, *tif*-1, *sfiA*-11, *uvrA*	Recombinant obtained from a cross: W2252 × GC714
KI211	APM^+, *tif*-1, *sfiA*-11, *uvrA*, pKM101	KI201 into which pKM101 was introduced
W2252	APM^+, HfrC	M. Ishibashi

[a] Ability to produce mutants forming large blue colonies on TGp-C-XP plates.

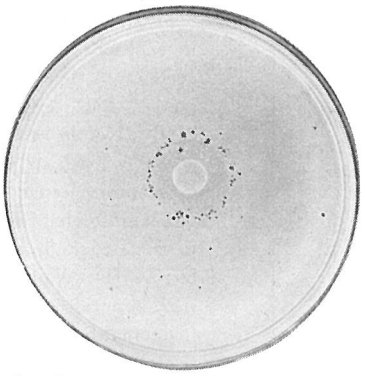


FIGURE 2. Spot test for APase constitutive mutation assay. 20 μl of MNNG was spotted on a filter disc in the center of a TGp-C-XP plate seeded with KI101 cells. Details of the assay procedure are described in the text. The mutant colonies appeared around the zone of inhibition.

Five chemicals (ethylene dibromide, *N*-nitrosomethylurea, MMS, NQNO and MNNG), which are all known to be mutagenic, were chosen for use in the assay. All of them produced APase constitutive mutants in the assay. The approximate sensitivities of the test strains are summarized in Table 2.

To test the validity of the assay and compare the sensitivities of the constructed strains, we carried out quantitative mutagenicity tests (Fig. 3A-C). Three mutagens, MNNG, MMS and NQNO, were selected for use in the assay because their modes of action were different. The mutagenic activities of the three chemicals were detected on assay with wild type strain KI101. With strains KI111 and KI211, pKM101 enhanced the mutagenic effects of all three chemicals. The mutagenic effects of the three chemicals were also enhanced by the *tif-1* and *uvrA* alleles, as shown in tests with KI201 and KI211. The relative sensitivities of the strains were com-

TABLE 2. *Qualitative mutation assay of various chemcals with the tester strains*

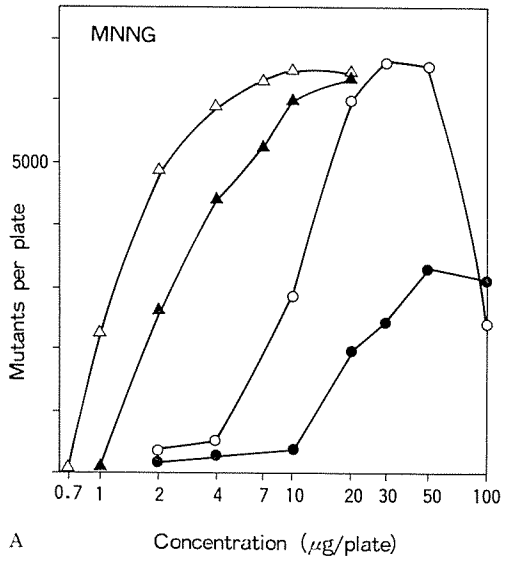
Chemical (applied to the paper disc)	Strain			
	KI101	KI111	KI201	KI211
Ethylene dibromide (20 μl)	−	±	+	+
Methyl methanesulfonate (5 μl)	+	+	+	+
4-Nitroquinoline-1-oxide (20 μg)	+	+	++	++
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (20 μg)	+	+	+	+
N-Nitrosomethylurea (10 μg)	+	+	+	++

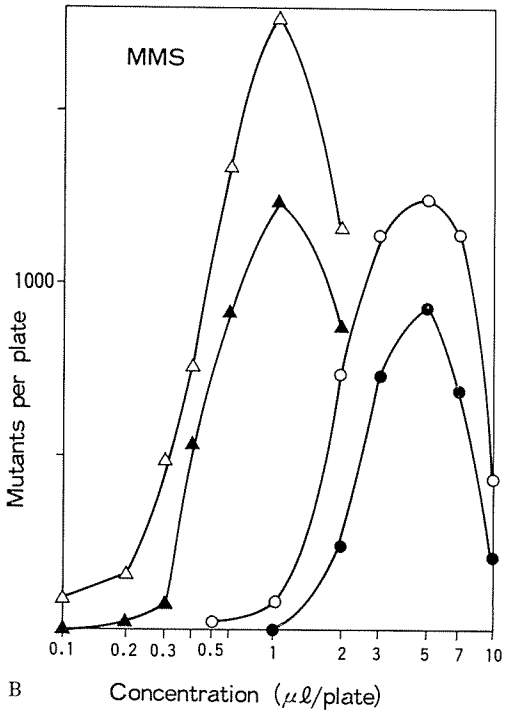
The assay procedure was as described in the text.
−: no detectable mutant colonies were formed.
±: a few mutant colonies were formed.
+: many mutant colonies were formed.
++: many mutant colonies were formed with less than the dose of mutagen described here.

TABLE 3. *Sensitivities of various strains to the three typical mutagens*

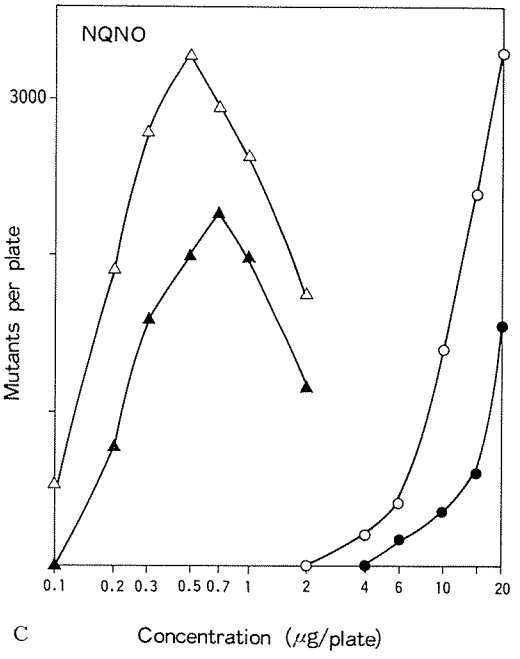
Chemical	Lowest effective dose[a]			
	KI101	KI111	KI201	KI211
N-Methyl-*N'*-notro-*N*-nitrosoguanidine (μg per plate)	7.8	3.8	0.96	0.68
Methyl methanesulfonate (μl per plate)	1.6	0.93	0.27	0.19
4-Nitroquinoline-1-oxide (μg per plate)	13	5.4	0.11	0.08

[a] The concentrarion corresponding to the intersection of the linear portion of the mutation curve extrapolated to the abscissa in Figs. 3A to C was arbitrarily defined as the lowest effective dose for mutagenesis.





pared by tests with the lowest effective doses of the chemicals for mutagenesis. As shown in Table 3, the sensitivities of the constructed strains in the assay decreased in the following order.



◀ ▲

FIGURE 3A-C. Comparison of relative sensitivities of the test strains to the representative mutagens, MNNG(A), MMS(B) and NQNO(C). APase constitutive mutation was measured as described in the text. The mean number of spontaneous mutant colonies per plate (125 for KI101, 69 for KI111, 149 for KI201 and 130 for KI211) was subtracted. Triplicate plates were used for each experiment. The concentrations of mutagens used in the present assay were within the range giving 10% survivors of each strain. The test strains were KI101 (wild) (●), KI111 (wild, pKM101) (○), KI201 (*tif*, *uvrA*) (▲), and KI211 (*tif*, *uvrA*, pKM101) (△).

KI211 (*tif*, *sfi*, *uvrA*, pKM101)>KI201 (*tif*, *sfi*, *uvrA*)>KI111 (pKM101)>KI101 (wild type).

DISCUSSION

In the present study we showed that APase constitutive mutations could be used for forward mutation assay. The sensitivity of the assay was considerably enhanced by introducing some genetic characters, such as *tif*, *uvrA* and pKM101, into the tester strains.

In the present assay, the mutants not only formed large colonies, but were also stained blue on a TGp-C-XP plate, and in this way colonies of true mutants could be identified. Another advantage of the assay is its simplicity, and its requirement for only one tester strain instead of the five needed in Ames' test (McCann et al., 1975b). Thus large numbers of chemicals can be screened by this spot test. In quantitative tests our tester strain, KI211, (table 3) showed the same order of sensitivity to three mutagens as observed with Ames' tester strains by McCann et al. (1975b).

We think that the APM^+ character is genetic, because it is transferable by conjugation and is stable. APM^- cells did not form large blue colonies on a TGp-C-XP plate containing mutagen, but they formed them on a TGp-C-XP plate when exposed to mutagen (MNNG) in liquid culture and then spread on the plate without mutagen. Among the strains examined, K10, W3747, W3623 and F-Silver were APM^+, while AB1157, from which JM1 and GC714 were derived, and AT2427 and JC411 were APM^-. No correlation was found between the APM^+ character and any known genetic marker, and no genealogical relationship was seen among the strains with the APM^+ character in the pedigree charts of Bachmann (1972). At present we have no explanation why APM^- strains do not respond to mutagens in the assay.

The mutagenicities of a few known mutagens (9-aminoacridine and proflavine) were not detected in the present assay. Since some chemicals do not readily penetrate into the cells, we expect that positive results could be obtained by introducing a permeability mutation, such as *tolC* or *envA*, into the tester strain (Otsuji et al., 1978; Moreau et al., 1976). Attempts are also being made to include a microsome activation system for assay of chemicals that need metabolic activation for mutagenesis.

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REFERENCES

Ames, B. N., Yanofsky, C. 1971. The detection of chemical mutagens with enteric bacteria, p. 267–282. *In* A. Hollaender [ed.] Chemical mutagens. vol. 1. Plenum Press, New York.

Ames, B. N., Lee, F. D., Durson, W. E. 1973. An improved bacterial test system for the detection of mutagens and carcinogens. Proc. Natl. Acad. Sci. USA 70: 782–786.

Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacterial. Rev. 36: 525–557.

Castellazzi, M., George, J., Buttin, G. 1972. Prophage induction and cell division in *E. coli*. 1. Further characterization of the thermosensitive mutation *tif-1* whose expression mimics the effect of UV irradiation. Mol. Gen. Genet. 119: 139–152.

George, J., Castellazzi, M., Buttin, G. 1975. Prophage induction and cell division in *E. coli*. III. Mutations *sfiA* and *sfiB* restore division in *tif* and *lon* strains and permit the expression of mutator properties of *tif*. Mol. Gen. Genet. 140: 309–332.

Kondo, S., Ichikawa, H., Iwo, K., Kato, T. 1970. Base-change mutagenesis and prophage induction in strains of *Eschericia coli* with different DNA repair capacities. Genetics 66: 187–217.

McCann, J., Spingarn, N. E., Kobori, J., Ames, B. N. 1975a. Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Natl. Acad. Sci. USA 72: 979–983.

McCann, J., Choi, E., Yamasaki, E., Ames, B. N. 1975b. Detection of carcinogens as mutagens in the *Salmonella* / microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72: 5135–5139.

Mohn, G. 1973. 5-Methyltryptophan resistance mutations in *Escherichia coli* K-12. Mutagenic activity of monofunctional alkylating agents including organophosphorus insecticides. Mutation Res. 20: 7–15.

Moreau, P., Bailone, A., Devoret, R. 1976. Pro-

phage λ induction in *Escherichia coli* K12 *envA uvrB*: A highly sensitive test for potential carcinogens. Proc. Natl. Acad. Sci. USA 73: 3700–3704.

Otsuji, N., Horiuchi, A., Nakata, A., Kawamata, J. 1978. Strains of *Escherichia coli* hypersensitive to representative carcinostatic and carcinogenic agents. J. Antibiot. (Tokyo) 31: 794–796.

Pueyo, C. 1978. Forward mutations to arabinose resistance in *Salmonella typhimurium* strains. Mutation Res. 54: 311–321.

Skopek, T. R., Liber, H. L., Krolewski, J. J., Thilly, W. G. 1978a. Quantitative forward mutation assay in *Salmonella typhimurium* using 8-azaguanine resistance as a genetic marker. Proc. Natl. Acad. Sci. USA 75: 410–414.

Skopek, T. R., Liber, H. L., Kadan, D. A., Thilly, W. G. 1978b. Relative sensitivities of forward and reverse mutation assays in *Salmonella typhomurium*. Proc. Natl. Acad. Sci. USA 75: 4465–4469.

Torriani, A., Rothman, F. 1961. Mutants of *Escherichia coli* constitutive for alkaline phosphatase. J. Bacteriol. 81: 835–836.

Walker, G. C. 1977. Plasmid (pKM101)-mediated enhancement of repair and mutagenesis: Dependence on chromosomal genes in *Escherichia coli* K-12. Mol. Gen. Genet. 152: 93–103.

Willsky, G. R., Bennett, R. L., Malamy, M. H. 1973. Inorganic phosphate transport in *Escherichia coli*: Involvement of two genes which play a role in alkaline phosphatase regulation. J. Bacteriol. 113: 529–539.