1. Introduction

Cellular DNA is continuously exposed to a variety of endogenous and exogenous genotoxic agents. Although DNA repair mechanisms are in operational to remove DNA lesions, DNA polymerases have to often encounter the lesions that are escaped from the repair mechanisms. DNA damages, such as ultraviolet light (UV) photoproducts or carcinogen adducts, strongly block the progress of DNA replication, and thus daughter-strand gaps are generated downstream of the lesions [1]. In *Escherichia coli*, the single-stranded regions are recognized by RecA protein, which mediates recombination to fill in the gaps with a homologous DNA sequence that is derived from the replicated sister chromatids [2]. In addition, the binding of RecA to the single-stranded regions activates RecA protein itself to mediate the cleavage of LexA repressor, which triggers the expression of more than 30 genes in the chromosome. This damage-inducible gene expression is referred to as an SOS response [3]. Interestingly, some of DNA polymerases in *E. coli* (see below) are regulated as part of the SOS response, and the SOS-inducible DNA polymerases appear to be involved in translesion DNA synthesis (TLS), which directly bypasses the lesions to fill in the gaps [4–6]. Some TLS reactions they catalyze are error-prone, i.e., incorporating incorrect bases in the nascent strand, while others are error free [7]. Thus, filling in the gaps by TLS appears to contribute to mutagenesis as well as to DNA damage tolerance, while filling-in reactions by homologous recombination are supposed to be non-mutagenic.

In *E. coli*, there are five DNA polymerases, i.e., DNA polymerases I–V [8]. DNA polymerase I encoded by *polA* is the first DNA polymerase to be described and is involved in lagging strand DNA synthesis, i.e., processing of Okazaki fragment, DNA repair and initiation of ColE1 plasmids such as pBR322 [9,10]. DNA polymerase II encoded by *polB* is a damage (SOS)-inducible DNA polymerase [11,12] and is involved in the process to restart the synthesis of damaged DNA [13,14]. This polymerase is reported to bypass 2-acetylaminofluorene adducts, which results in −2 frameshift [15,16]. Unlike DNA polymerase I, which belongs to A-family DNA polymerase, DNA polymerase II is a member of B family, in which mammalian replicative DNA polymerases such as DNA polymerase delta are included [17]. DNA polymerase III holoenzyme, which is composed of multiple subunits, is responsible for the chromosome replication of *E. coli* and the catalytic subunit is encoded by *dnaE* (or *polC*) [18,19]. This enzyme is classified into C family, in which prokaryotic replicative DNA polymerases are categorized [8]. DNA polymerase IV and DNA polymerase V belong to Y family, whose members are mostly involved in TLS, and the expression of *dinB* and *umuDC* encoding DNA polymerase IV and DNA polymerase V, respectively, is regulated as part of the SOS response [20–24]. DNA polymerase IV is shown to be involved in −1 frameshift mutagenesis induced by 4-nitroquinoline N-oxide and benzo[a]pyrene [25,26], and DNA polymerase V is known to play important roles in mutagenesis induced by UV and a variety of genotoxic compounds [27,28]. However, current knowledge about the roles of replicative, i.e., DNA polymerase I and DNA polymerase III, and SOS-inducible specialized DNA polymerases, i.e., DNA polymerase II, DNA polymerase IV and DNA polymerase V, in mutagenesis is still limited because synthetic oligonucleotides bearing specific DNA lesions are required for in vitro and in vivo analyses to address the question.

Salmonella typhimurium is a Gram negative bacterium, whose genome sequence is 70–90% homologous to *E. coli* [29]. Some of *S. typhimurium* strains have been widely used to detect a variety of environmental mutagens and carcinogens as tester strains of Ames test [30]. One of such strain TA1538 possesses CGCGCGCG sequence in the *hisD* gene, which is a mutational hot spot for −2 (–CG) frameshift [31,32]. The strain bears a deep-rough *rfa* mutation, which increases the permeability to hydrophobic compounds such as polycyclic aromatic hydrocarbons (PAH) [33]. In addition, the strain is deficient in the capacity to excise bulky DNA adducts by the *uvrB* mutation, so that the DNA adducts are more likely to be bypassed rather than removed by repair enzymes [33].

In a previous study, we have systematically disrupted the genes of *S. typhimurium* TA1538 encoding SOS-inducible DNA polymerases, i.e., $polB_{ST}$, $dinB_{ST}$, $umuDC_{ST}$ and *samAB*, and concluded that different sets of DNA polymerases are engaged in lesion bypass in the CGCGCGCG sequence depending upon the environmental threats by chemicals [34]. We also proposed that not only SOS-inducible DNA polymerases but also the main replicative DNA polymerase, i.e., DNA polymerase III, plays important roles in −2 frameshift [34].

In this study, we generated a set of isogenic derivatives of *S. typhimurium* TA1538 by introducing plasmids carrying *polB*, *dinB* or *mucAB* encoding *E. coli* DNA polymerase II, DNA polymerase IV or DNA polymerase RI, respectively, and examined the mutability to 30 chemicals. We introduced the plasmid carrying *mucAB*, i.e., pKM101, instead of a plasmid carrying *E. coli umuDC*, because DNA polymerase RI is a homologue of *E. coli* DNA polymerase V [35], and the derivative of TA1538 harboring plasmid pKM101, i.e., strain TA98, has been widely used as a standard tester strain of Ames test [30]. We also introduced a plasmid carrying the *polA* gene of *E. coli* to strain TA1538 and examined the mutability to investigate the possible involvement of DNA polymerase I in TLS leading to frameshift. Intriguingly, the introduction of the *polA* plasmid completely suppressed the mutations depending on the activities of $dinB_{ST}$ and $umuDC_{ST}$ of *S. typhimurium*. Collectively, the present results suggest that (1) DNA polymerase IV and DNA polymerase RI has distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, (2) the replicative DNA polymerase, i.e., DNA polymerase III, substantially contributes to −2 frameshift and (3) the enhanced expression of *E. coli* polymerase I inhibits the access of Y-family DNA polymerases to the replication complex where TLS occurs. In addition, our results raise an interesting possibility that strain YG5161 harboring plasmid pYG768 carrying *dinB* could be a superior tester strain to strain TA98 to detect the mutagenicity of environmental PAHs.

2. Materials and methods

2.1. *Strains and plasmids*

The strains and plasmids used in this study are listed in Table 1. Strain YG5160 and strain YG5161 were constructed

Table 1 – *S. typhimurium* strains and plasmids

Strain or plasmid	Description	Source
Strains		
TA1535	*hisG46, gal, Δ (chl, uvrB, bio), rfa*	Maron and Ames [30]
TA1537	*hisC3076, gal, Δ (chl, uvrB, bio), rfa*	Maron and Ames [30]
TA1538	*hisD3052, gal, Δ (chl, uvrB, bio), rfa*	Maron and Ames [30]
TA98	As TA1538 but harbors plasmid pKM101	Maron and Ames [30]
YG5160	As TA1538 but harbors plasmid pYG787	This study
YG5161	As TA1538 but harbors plasmid pYG768	This study
YG6215	As TA1538 but $\Delta umuDC_{ST}$::Km^r, $\Delta samAB$::Cm^r, $\Delta dinB_{ST}$::Sp^r, $\Delta polB_{ST}$::Tc^r	Kokubo et al. [34]
Plasmids		
pKM101	Plasmid carrying the *mucAB* genes	Maron and Ames [30]
pYG768	Derivative of pWSK29 with the *E. coli dinB* gene	Kim et al. [36]
pYG787	Derivative of pWKS30 with the *E. coli polB* gene	Kokubo et al. [34]
pIMA-1	Derivative of pWKS30 with the *E. coli polA* gene	Imai and Yamamoto (unpublished)

by introduction of plasmid pYG787 carrying *polB* and plasmid pYG768 carrying *dinB*, respectively, into strain TA1538 [34,36]. Plasmid pIMA-1 carrying *E. coli polA* [9] was constructed by the insertion of a 3.5-kb fragment of the *polA* gene between EcoRI and SalI sites of plasmid pWKS30 (Imai and Yamamoto, unpublished). The direction of transcription of the *polA* gene in the plasmid is opposite to that of the *lacZ* gene. The plasmid could complement the killing sensitivity of a *polA* strain of *E. coli* to ultraviolet light and methyl methanesulfonate. Transformation was conducted by electroporation [37].

2.2. Chemicals

The names, abbreviations, CAS registry numbers and sources of the chemicals used in this study are listed in Table 2. The chemical structures are presented in Fig. 1.

2.3. Media

Luria–Bertani broth and agar were used for bacterial culture [38]. Vogel–Bonner minimal agar plates and top agar were prepared as previously described, and used for the His^+ reversion assay with *S. typhimurium* [30]. Nutrient broth (Difco, MI, USA) with ampicillin (AP, 25 μg/ml) was used for pre-cultures of the strains for the reversion assay.

2.4. Mutagenicity assay

The mutagenicity assay was carried out with a pre-incubation procedure [30]. Briefly, 0.1 ml overnight culture was incubated with the chemicals dissolved in 0.1 ml solvent and 0.5 ml S9 mix for 20 min at 37 °C. When S9 mix is not required, 0.5 ml of 1/15 M phosphate buffer pH 7.4 was added. The mixture was then poured onto agar plates with soft agar and incubated for 2 days at 37 °C. Each chemical was assayed with four to seven doses on duplicate plates with four strains, i.e., strain TA1538, TA98, YG5160 and YG5161, in parallel. In the series of experiments, we regarded the effects of introduction of plasmids, i.e., pYG787, pYG768 or pKM101, on the mutability of strain T1538 as significant when the transformed strains displayed more than and including 50% higher or lower mutability, compared to the parent strain TA1538.

3. Results

3.1. Specificity of SOS-inducible DNA polymerases in frameshift induced by 30 chemicals

To assign the role of SOS-inducible DNA polymerases in bypass of DNA lesions, we introduced plasmids carrying *E. coli polB*, *dinB* or *mucAB* encoding different SOS-inducible DNA polymerases to strain TA1538 and examined their mutability to 30 chemicals. The dose–response curves are presented in Fig. 2, and the numbers of revertants per microgram per plate of each chemical and strain are summarized in Table 3. To make the comparison easier, we also calculated the relative mutability of each derivative by assigning the number of revertants per microgram in strain TA1538 as 1.0. According to the mutagenicity, we classified the 30 chemicals into four groups as follows.

Group I includes benzo[a]pyrene and other seven chemicals. The mutagenicity of these compounds was highest in strain YG5161 harboring plasmid pYG768 carrying *dinB* encoding DNA polymerase IV, followed by strain TA98 harboring plasmid pKM101 carrying *mucAB* encoding DNA polymerase RI. The mutagenicity of the chemicals to strain YG5160 harboring plasmid pYG787 carrying *polB* encoding DNA polymerase II was very similar to the parent strain TA1538 except for benzo[a]pyrene-7,8-dihydroepoxide and 1-aminoanthracene where introduction of plasmid pYG787 appeared to alleviate the mutagenicity by 50% and 40%, respectively. For benzo[a]pyrene, the ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 7:2:1:1. The compounds in this group are derivatives of benzo[a]pyrene except for 3-methylcholanthrene, 1-aminoanthracene and 2-aminoanthracene.

Group II includes ENNG and other four chemicals. The mutagenicity of these compounds was almost equally high in strain YG5161 and strain TA98. The introduction of plasmid pYG787 carrying *polB* did not enhance the mutagenicity. Rather, plasmid pYG787 seemed to reduce the mutagenicity of 6-aminochrysene by 60%. The ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 20:19:1:1 for ENNG. The compounds in this group are PAHs and the derivative except for ENNG.

Table 2 – Names, abbreviations, CAS registry numbers and sources of the chemicals

Chem. no.	Chemical	CAS registry numbers	Sources[a]
1	Benzo[a]pyrene-7,8-dihydroepoxide	36504-65-1	Mi
2	Benzo[a]pyrene diol epoxide	58917-67-2	Mi
3	10-Azabenzo[a]pyrene	189-92-4	1
4	Benzo[a]pyrene	50-32-8	W
5	3-Nitro-benzo[a]pyrene	70021-98-6	4
6	3-Methylcholanthrene	56-49-5	S
7	1-Aminoanthracene	610-49-1	S
8	2-Aminoanthracene	613-13-8	W
9	7,12-Dimethylbenz[a]anthracene (DMBA)	57-97-6	W
10	6-Aminochrysene	2642-98-0	S
11	1-Nitro-benzo[a]pyrene	70021-99-7	4
12	Benzo[a]pyrene-4,5-dihydroepoxide	64437-52-1	Mi
13	N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	4245-77-6	5
14	1-Nitropyrene	5522-43-0	T
15	1,8-Dinitropyrene	42397-65-9	T
16	6-Nitro-benzo[a]pyrene	63041-90-7	4
17	1-Nitro-6-azabenzo[a]pyrene	138835-35-5	4
18	3-Nitro-6-azabenzo[a]pyrene	138835-36-6	4
19	Furylfuramide	3688-53-7	W
20	Aflatoxin B1	1162-65-8	S
21	Benzo[a]pyrene-7,8-tetrahydroepoxide	36504-67-3	Mi
22	Acridine orange	65-61-2	Me
23	Benz[a]anthracene	56-55-3	S
24	2-Nitrofluorene	607-57-8	T
25	2-[2-(Acetylamino)-4-[bis-(2-methoxy-ethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1)	194590-84-6	3
26	2-Amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1)	67730-11-4	W
27	Aminophenylnorharman	219959-86-1	3
28	N-Hydroxyacetylaminofluorene (N-OH-AAF)	53-95-2	Mi
29	4-Nitroquinoline-1-oxide (4-NQO)	56-57-5	T
30	2-Acetylaminofluorene	53-96-3	T

[a] The chemicals were purchased from the following sources at the highest grade of purity: Wako Pure Chemical (W); Tokyo Kasei Kogyo (T); Sigma–Aldrich (S); Merck (Me); Midwest Research Institute (Mi); Nacalai Tesque (N). Commercially unavailable chemicals were provided by the follwing persons: Dr. Ken-ichi Saeki, Nagoya City University, Japan (1); Dr. Takeji Takamura-Enya, National Cancer Center Research Institute, Tokyo, Japan (2); Dr. Yukari Totsuka, National Cancer Center Research Institute, Tokyo, Japan (3); Dr. Kiyoshi Fukuhara, National Institute of Health Sciences, Tokyo, Japan (4); laboratory stock (5).

Group III includes 1-nitropyrene and other 10 chemicals. The mutagenicity of these compounds was highest in strain TA98. Introduction of plasmid pYG768 carrying *dinB* displayed moderate (less than three-fold) enhancing effects on the mutagenicity of this group of chemicals. The introduction of plasmid pYG787 carrying *polB* enhanced the mutagenicity of benz[a]anthracene three-fold reproducibly, although it had no enhancing effects on other chemicals. For the mutagenicity of 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and furylfuramide, plasmid pYG787 reduced the mutagenicity by half. The ratio of the mutability of strain TA98, YG5161, YG5160 and TA1538 was 16:1:1:1 for 1-nitropyrene. The compounds in this group include structurally unrelated compounds such as furylfuramide, aflatoxin B1 and acridine orange.

Group IV includes 2-acetylaminofluorene and other five compounds. The characteristic of this group was that the mutagenicity was not enhanced by the introduction of any of the plasmids encoding SOS-inducible DNA polymerases. The ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 1:1:1:1 for 2-acetylaminofluorene. The compounds in this group are aromatic amines except for 4-NQO.

3.2. −1 *Frameshit and base substitutions by benzo[a]pyrene and ENNG promoted by DNA polymerase IV and DNA polymerase RI*

Since DNA polymerase IV encoded by *dinB* appeared to promote −2 frameshift induced by benzo[a]pyrene (group I chemical) and ENNG (group II chemical), we examined the possibility whether the polymerase also promotes other types of mutations, i.e., −1 frameshift and base substitutions, by the chemicals. To this end, we took advantage of other *S. typhimurium* strains, i.e., TA1537 and TA1535, which detects mutagens that cause −1 frameshift in CCC sequence in the *hisC* gene and base substitutions in GGG sequence in the *hisG* gene, respectively [30]. For benzo[a]pyrene-induced mutagenesis, the introduction of plasmid pYG768 carrying *dinB* into strain TA1537 slightly enhanced the mutagenicity, but the effect of enhancing mutagenesis was much lower compared to the effect of plasmid pKM101 carrying *mucAB* encoding DNA polymerase RI (Fig. 3A). For base substitutions, DNA polymerase IV seemed inactive and virtually no enhancement was observed in strain TA1535 with plasmid pYG768. In contrast, DNA polymerase RI actively promoted the base substitution mutations. As has been observed

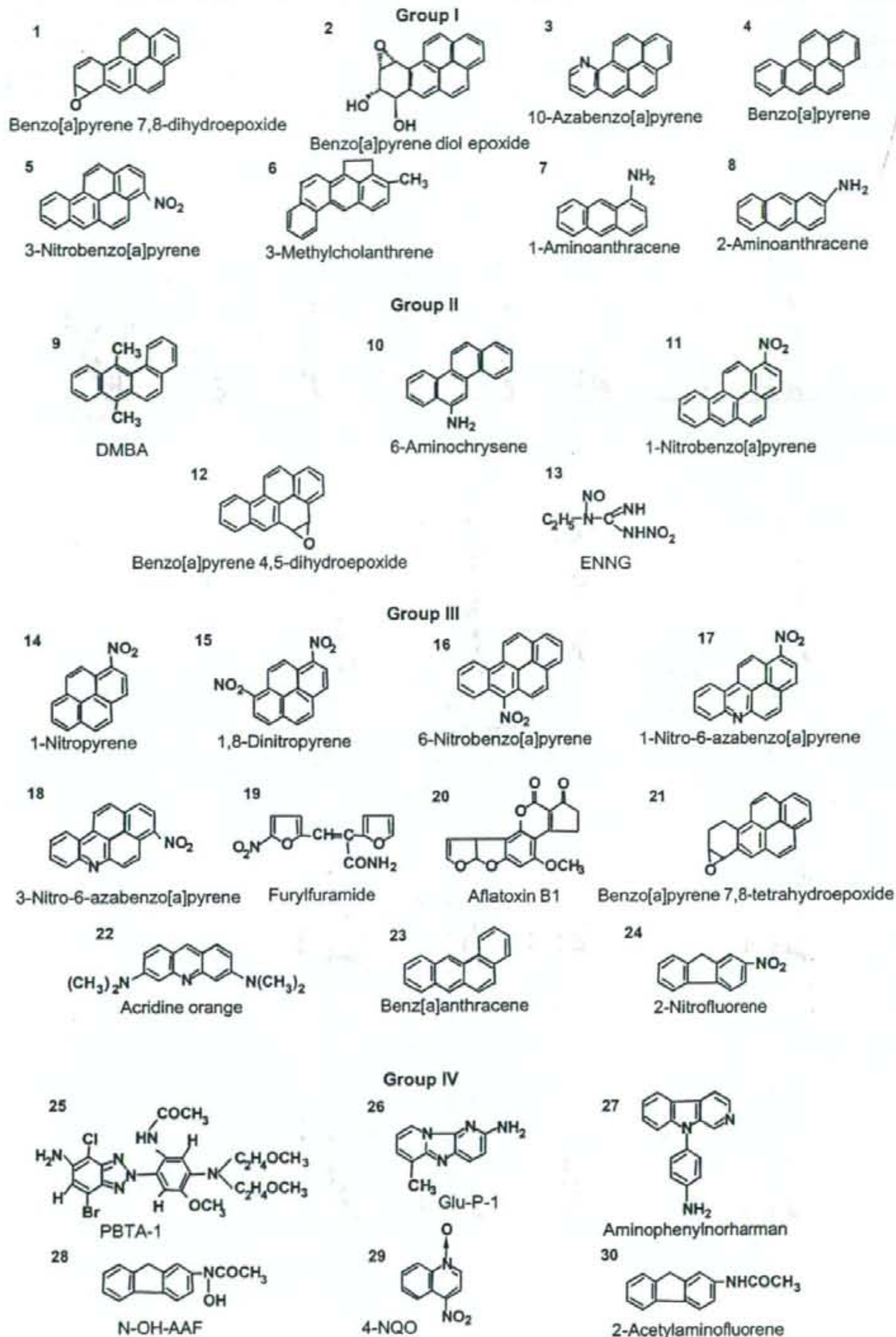

Fig. 1 – Structures of the chemical mutagens used in this study.

in −2 frameshift, introduction of plasmid pYG787 carrying *polB* had almost no effects on any types of mutations induced by benzo[a]pyrene. These results suggest that the efficiency of error-prone bypass across lesions by DNA polymerase IV strongly depends on the types of mutations and the sequence context surrounding the lesions. For ENNG-induced mutagenesis, both DNA polymerase IV and DNA polymerase RI appeared to promote −1 frameshift and base

substitutions substantially (Fig. 3B). Introduction of plasmid pYG787 carrying *polB* had almost no effects on the mutability. Unlike ENNG-induced mutagenesis, −2 frameshift, −1 frameshift and base substitutions induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were not enhanced by the introduction of either plasmid pYG768 or pKM101 (data not shown). These results suggest that both DNA polymerase IV and DNA polymerase RI bypass ethyl, but not methyl, adducts in DNA leading to −2 and −1 frameshifts and base substitutions.

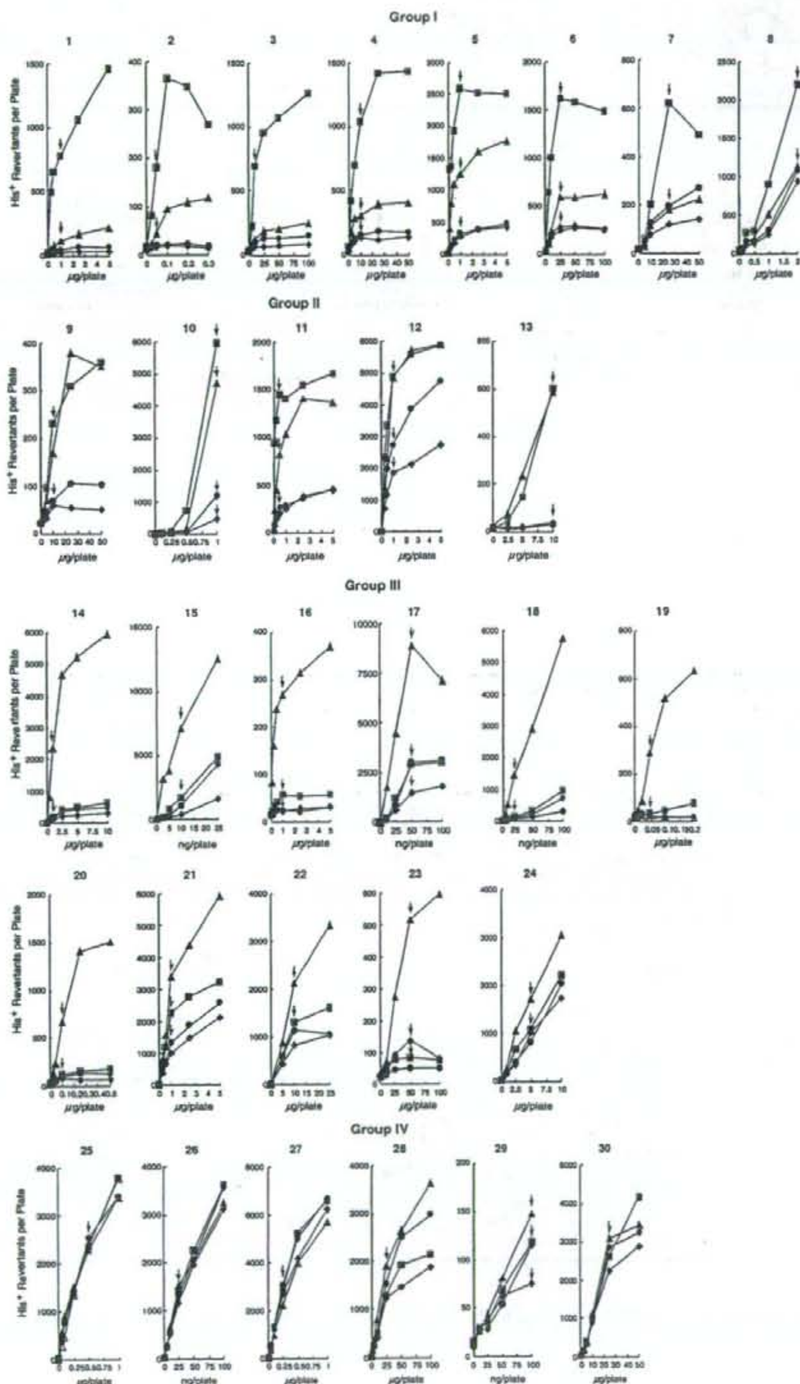

Table 3 – Mutagenicity of 30 chemicals in *S. typhimurium* strains harboring plasmids carrying genes encoding *E. coli* SOS DNA polymerases

Number	Chemical	Group	S9	TA1538 no plasmid	TA98 pKM101 (DNA pol RI)	YG5160 pYG787 (DNA pol II)	YG5161 pYG768 (DNA pol IV)
1	Benzo[a]pyrene-7,8-dihydroepoxide	I	–	44 (1.0)	113 (2.6)	22 (0.5)	776 (17.6)
2	Benzo[a]pyrene diol epoxide	I	–	420 (1.0)	920 (2.2)	320 (0.8)	3620 (8.6)
3	10-Azabenz[a]pyrene	I	+	9 (1.0)	12 (1.3)	6 (0.7)	68 (7.6)
4	Benzo[a]pyrene	I	+	15 (1.0)	30 (2.0)	13 (0.9)	104 (6.9)
5	3-Nitro-benzo[a]pyrene	I	–	316 (1.0)	1244 (3.9)	277 (0.9)	2580 (8.2)
6	3-Methylcholanthrene	I	+	10 (1.0)	23 (2.3)	11 (1.1)	65 (6.5)
7	1-Aminoanthracene	I	+	8 (1.0)	7 (0.9)	5 (0.6)	25 (3.1)
8	2-Aminoanthracene	I	+	540 (1.0)	564 (1.0)	468 (0.9)	1102 (2.0)
9	DMBA	II	+	7 (1.0)	17 (2.4)	6 (0.9)	23 (3.3)
10	6-Aminochrysene	II	+	1200 (1.0)	4693 (3.9)	461 (0.4)	5955 (5.0)
11	1-Nitro-benzo[a]pyrene	II	–	524 (1.0)	1640 (3.1)	400 (0.8)	2896 (5.5)
12	Benzo[a]pyrene-4,5-dihydroepoxide	II	–	2724 (1.0)	4836 (1.8)	1856 (0.7)	4900 (1.8)
13	ENNG	II	–	3 (1.0)	58 (19.3)	4 (1.3)	60 (20)
14	1-Nitropyrene	III	–	154 (1.0)	2354 (15.3)	112 (0.7)	194 (1.3)
15	1,8-Dinitropyrene	III	–	110100 (1.0)	708300 (6.4)	39300 (0.4)	171600 (1.6)
16	6-Nitro-benzo[a]pyrene	III	–	24 (1.0)	268 (11.2)	21 (0.9)	58 (2.4)
17	1-Nitro-6-azabenzo[a]pyrene	III	–	60500 (1.0)	178180 (2.9)	29700 (0.5)	58020 (1.0)
18	3-Nitro-6-azabenzo[a]pyrene	III	–	3560 (1.0)	57640 (16.2)	2480 (0.7)	5240 (1.5)
19	Fulylfuramide	III	–	480 (1.0)	5760 (12.0)	220 (0.5)	620 (1.3)
20	Aflatoxin B1	III	+	990 (1.0)	6680 (6.7)	750 (0.8)	1160 (1.2)
21	Benzo[a]pyrene-7,8-tetrahydroepoxide	III	–	1332 (1.0)	3404 (2.6)	1000 (0.8)	2252 (1.7)
22	Acridine orange	III	+	113 (1.0)	234 (2.1)	83 (0.7)	131 (1.2)
23	Benz[a]anthracene	III	+	1 (1.0)	10 (10.0)	3 (3.0)	2 (2.0)
24	2-Nitrofluorene	III	–	162 (1.0)	341 (2.1)	203 (1.3)	215 (1.3)
25	PBTA-1	IV	+	5074 (1.0)	4568 (0.9)	4768 (0.9)	4720 (0.9)
26	Glu-P-1	IV	+	52800 (1.0)	61120 (1.2)	46480 (0.9)	56640 (1.1)
27	Aminophenylnorharman	IV	+	12352 (1.0)	8880 (0.7)	10688 (0.9)	11456 (0.9)
28	N-OH-AAF	IV	–	62 (1.0)	76 (1.2)	49 (0.8)	52 (0.8)
29	4-NQO	IV	–	1150 (1.0)	1470 (1.3)	750 (0.7)	1180 (1.0)
30	2-Acetylaminofluorene	IV	+	114 (1.0)	124 (1.1)	89 (0.8)	105 (0.9)

Each chemical was assayed with four to seven doses on duplicate plates with four strains in parallel. The assays with chemical nos. 2, 4, 7, 8, 10, 12, 15, 17, 23 and 29 were repeated to confirm the initial results. The numbers of His$^+$ revertants per plate per microgram of each strain are calculated at the doses indicated with arrows in Fig. 2. The numbers in parentheses represent the values relative to the numbers of His$^+$ revertants per microgram in TA1538 (no plasmid). Difference of the relative mutability two-fold or more was regarded as significant effects of the introduction of plasmids on the mutability.

Group I: the chemicals whose mutagenicity was highest in strain YG5161 harboring plasmid pYG768 carrying *dinB* (DNA pol IV).

Group II: the chemicals whose mutagenicity was equally high in both strain YG5161and strain TA98 harboring plasmid pKM101 carrying *mucAB* (DNA pol RI).

Group III: the chemicals whose mutagenicity was highest in strain TA98.

Group IV: the chemicals whose mutagenicity was not substantially modulated by the introduction of any of the plasmids.

Fig. 2 – Responses of *S. typhimurium* tester strains to 30 chemical mutagens. The chemicals are: benzo[a]pyrene-7,8-dihydroepoxide (1); benzo[a]pyrene diol epoxide (2); 10-azabenzo[a]pyrene (3); benzo[a]pyrene (4); 3-nitro-benzo[a]pyrene (5); 3-methylcholanthrene (6); 1-aminoanthracene (7); 2-aminoanthracene (8); DMBA (9); 6-aminochrysene (10); 1-nitro-benzo[a]pyrene (11); benzo[a]pyrene-4,5-dihydroepoxide (12); ENNG (13); 1-nitropyrene (14); 1,8-dinitropyrene (15); 6-nitro-benzo[a]pyrene (16); 1-nitro-6-azabenzo[a]pyrene (17); 3-nitro-6-azabenzo[a]pyrene (18); furylfuramide (19); aflatoxin B1 (20); benzo[a]pyrene-7,8-tetrahydroepoxide (21); acridine orange (22); benz[a]anthracene (23); 2-nitrofluorene (24); PBTA-1 (25); Glu-P-1 (26); aminophenylnorharman (27); N-OH-AAF (28); 4-NQO (29); 2-acetylaminofluorene (30). The strains used are: TA1538 (circles ●); YG5160 (diamonds ♦); YG5161 (squares ■); TA98 (triangles ▲). The arrow indicates the dose that was used for the calculation of His$^+$ revertants per microgram per plate in Table 3.

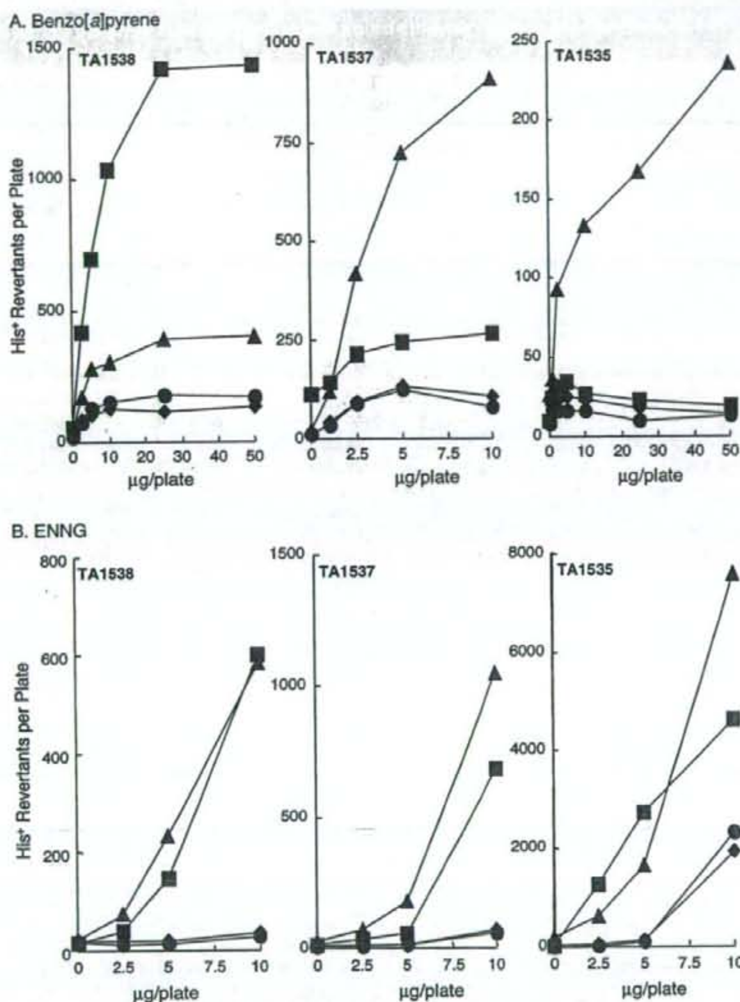

Fig. 3 – Responses of S. *typhimurium* tester strains to benzo[a]pyrene and ENNG. Benzo[a]pyrene plus S9 mix (A) or ENNG (B) was assayed with four to six doses on duplicate plates with four strains, i.e., the parent strain without plasmids (circles ●), the derivative harboring plasmid pYG787 carrying *polB* (diamonds ♦), the derivative harboring plasmid pYG768 carrying *dinB* (squares ■) and the derivative harboring plasmid pKM101 carrying *mucAB* (triangles ▲) in parallel. The parent strains were S. *typhimurium* TA1538, TA1537 and TA1535 for the detection of −2 frameshift, −1 frameshift and base substitutions, respectively, and they were transformed with one of three plasmids, i.e., pYG787, pYG768 and pKM101.

3.3. *Effects of the introduction of plasmid carrying polA encoding E. coli DNA polymerase I on the mutability of strain TA1538*

The mutagenicity of the compounds of group IV was not influenced by introduction of any of the plasmids carrying genes encoding SOS-inducible DNA polymerases (Fig. 2). This suggests the involvement of replicative DNA polymerases, i.e., DNA polymerase I and/or DNA polymerase III, in the mutagenesis. To examine the possible involvement of DNA polymerase I, we introduced plasmid pIMA-1 carrying *polA* encoding DNA polymerase I to strain TA1538 and its derivative YG6215, which lacks all the genes encoding SOS-inducible DNA polymerases [34], and compared the mutability to the group IV compounds, i.e., PBTA-1, Glu-P-1, aminophenylnorharman, 4-NQO and 2-acetylaminofluorene (Fig. 4). We also examined the mutagenicity of 2-aminofluorene, a derivative of 2-acetylaminofluorene. The introduction of plasmid pIMA-1 did not affect the mutability of strain TA1538 and YG6215 to the group IV compounds and 2-aminofluorene. We also examined the mutability of strain TA1538 and YG6215 harboring plasmid pIMA-1 carrying *polA* to other chemicals belong to group I, i.e., benzo[a]pyrene, 10-azabenzo[a]pyrene, 3-methylcholanthrene and 1-aminoanthracene, group II,

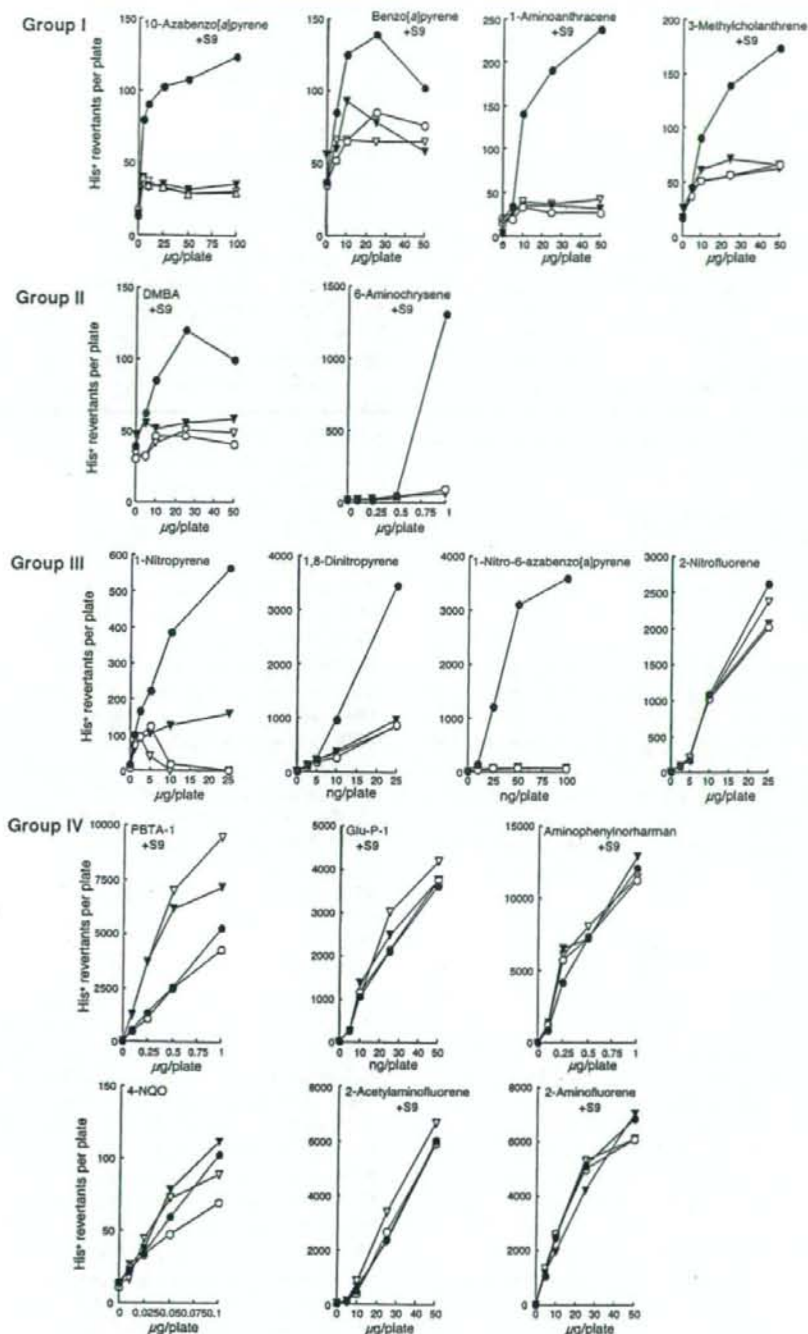

Fig. 4 – Effects of introduction of plasmid pIMA-1 carrying *polA* on the mutability of strain TA1538 and YG6215 to chemicals. The strains used were *S. typhimurium* TA1538 harboring the vector pWKS30 (closed circles ●), TA1538 harboring plasmid pIMA-1 (open circles ○), YG6215 harboring the vector pWKS30 (closed triangles ▼) and YG6215 harboring plasmid pIMA-1 (open triangles ▽). Each chemical was assayed with five to six doses on triplicate plates with four strains in parallel. The dose response curves of PBTA-1 plus S9 was unique in that strain YG6215 lacking all SOS-inducible DNA polymerases displayed higher mutability than strain TA1538 [34].

i.e., DMBA and 6-aminochrysene and group III, i.e., 1-nitropyrene, 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and 2-nitrofluorene. Surprisingly, the introduction of plasmid pIMA-1 reduced the mutability of strain TA1538 to the level of strain YG6215 harboring the vector plasmid (Fig. 4). Only exception was 2-nitrofluorene where the mutability of strain TA1538 and YG6215 was not affected by the introduction of plasmid pIMA-1 as in the case of group IV compounds. The dose–response curves of strain YA1538 harboring plasmid pIMA-1 almost completely overlapped those of strain YG6215 harboring the vector plasmid. The mutability of strain YG6215 was not affected by the introduction of plasmid pIMA-1 carrying *polA* except for 1-nitropyrene where strain YG6215 as well as strain TA1538 exhibited an enhanced killing sensitivity to the chemical when the *polA* plasmid was introduced.

4. Discussion

DNA polymerase III holoenzyme replicates the chromosome of *E. coli* with high fidelity but its progress is occasionally blocked by DNA lesions, and DNA polymerase V encoded by *umuDC* bypasses the lesions to assist the chromosome replication, which mostly results in base substitutions [10,28]. Less is known, however, about the mechanisms of translesion bypass leading to frameshift [39–41]. To assign the role of each SOS-inducible DNA polymerases of *E. coli* in TLS leading to frameshift, we have introduced plasmids carrying the genes encoding SOS-inducible DNA polymerases to strain TA1538 possessing CGCGCGCG sequence in the *hisD* gene as a −2 frameshift hot spot [31], and examined the mutability to 30 chemical mutagens. The *mucB* gene is expressed 13,000 molecules per cell from plasmid pKM101 when LexA repressor is inactivated [42], and the expression level is much higher than the chromosomal expression level of the *umuC* gene, i.e., about 200 molecules per cell upon SOS induction [43]. Similarly, the *dinB* gene is expressed 25,000–50,000 molecules per cell from plasmid pYG768 in the presence of DNA damage, which is 10–20 times higher than the expression level of *dinB* from the chromosome upon SOS induction [25]. The exact expression levels of DNA polymerase I and DNA polymerase II from plasmid pIMA-1 and pYG787, respectively, are unclear, but they could be at least two to three times higher than those from the chromosome because both plasmids are derivatives of low-copy-number plasmid pWKS30 [44]. DNA polymerase I and DNA polymerase II encoded by *polA* and *polB*, respectively, are expressed 400 and 30–50 molecules per cell from the chromosome and the expression of *polB* is enhanced about seven-fold by DNA damage [10,45]. Thus, we assume the expression levels are about 1000 for DNA polymerase I, 500–1000 for DNA polymerase II, 10–20 for DNA polymerase III [10], 25,000–50,000 for DNA polymerase IV and 13,000 for DNA polymerase RI per cell when the chromosome DNA is damaged by chemicals. Because of the high levels of expression of these DNA polymerases from the plasmids, we could observe distinct enhancing effects on the mutability of strain TA1538 to 30 chemicals (Fig. 2, Table 3). Based on the mutagenicity, we classified the chemicals into four groups as follows.

Group I includes eight chemicals that exhibited highest mutagenicity in strain YG5161 harboring plasmid pYG768 carrying *dinB*. The introduction of plasmid pKM101 also enhanced the mutagenicity of some compounds, but the extent of the enhancement was much less compared to the effects of plasmid pYG768. In fact, the introduction of plasmid pKM101 into strain TA1538 did not enhance the mutagenicity of 1-aminoanthracene and 2-aminoanthracene while plasmid pYG768 enhanced the mutagenicity more than two times. These results suggest that DNA polymerase IV encoded by *dinB* efficiently bypasses DNA lesions induced by group I compounds in CGCGCGCG sequence leading to −2 frameshift. This is consistent with our previous results that deletion of endogenous *dinB* gene of *S. typhimurium*, i.e., $dinB_{ST}$, significantly reduced the mutability of strain TA1538 to 10-azabenzo[a]pyrene, benzo[a]pyrene, 1-aminoanthracene and 2-aminoanthracene [34]. DNA polymerase IV could have specificity to bypass across guanine bases modified with the polycyclic aromatics, leading to −2 frameshift. In contrast to −2 frameshift, the introduction of plasmid pYG768 did not enhance base substitutions induced by benzo[a]pyrene (Fig. 3A). *E. coli* DNA polymerase IV is reported to bypass N^2-guanine adducts of benzo[a]pyrene diol epoxide in vitro with higher efficiency and high fidelity compared to DNA polymerase II and DNA polymerase V [46]. Thus, we suggest that DNA polymerase IV correctly incorporate cytosine opposite the adducted guanine in the GGG sequence in the *hisG* gene, thereby leading to no base substitutions. Correct insertion of cytosine opposite the adducted guanine in the CGCGCGCG sequence in *hisD*, however, may induce a two-bp slippage leading to −2 frameshift [47].

Group II includes five chemicals that displayed equally high mutagenicity to strain YG5161 and strain TA98. They are derivatives of PAHs, i.e., DMBA and benzo[a]pyrene-4,5-dihydroepoxide, an aromatic amine, i.e., 6-aminochrysene, a nitroaromatic, i.e., 1-nitrobenzo[a]pyrene and an alkylating agent, i.e., ENNG. The results that strains YG5161 and TA98 exhibited similar mutability to the compounds suggest that lesions induced by group II compounds can be bypassed by either DNA polymerase IV or DNA polymerase RI at similar efficiency. In fact, the previous study indicates that deletion of either $dinB_{ST}$ or $umuDC_{ST}$ reduced the mutagenicity of DMBA and 6-aminochrysene [34]. These compounds appear to require the presence of at least two specialized DNA polymerases to bypass the lesions. Of group II compounds, ENNG is exceptional because it is a simple alkylating agent that induces a mutagenic guanine base, i.e., O^6-ethylguanine [48]. Since the lesion in DNA directs the incorporation of thymine as well as cytosine during DNA synthesis, it was expected that this compound enhanced base substitutions in strain TA1535 (Fig. 3 B). It was a big surprise, however, that the simple alkylating agent was also capable of inducing −1 and −2 frameshifts in the repetitive sequences in strains TA1537 and TA1538, respectively, and that the bypass reactions leading to frameshifts appeared to be mediated by DNA polymerase IV or DNA polymerase RI. We also observed that the introduction of plasmids pYG768 and pKM101 enhanced the mutability of strain TA1538 against ethylnitrosourea (ENU), which induces O^6-ethylguanine in DNA (unpublished results). Eckert and Hile reported that frameshift errors are generated during in vitro DNA synthesis of ENU-treated template single-stranded DNA by mammalian DNA alpha-primase and DNA polymerase beta

[49]. Since humans possess Y-family DNA polymerases, i.e., DNA polymerase eta, iota, kappa and REV1 [8], it seems worth examining the abilities to bypass O^6-ethylguanine in repetitive sequences leading to frameshifts.

Since the newly established strain YG5161 harboring plasmid pYG768 exhibited higher sensitivity to groups I and II compounds than the standard Ames tester strain TA98 harboring plasmid pKM101, strain YG5161 could be useful for the sensitive detection of environmental mutagens and carcinogens such as benzo[a]pyrene and its derivatives. Actually, 10-azabenzo[a]pyrene, benzo[a]pyrene and 3-nitrobenzo[a]pyrene are present in polluted air and soot of combustion of coal [50,51], and some of them are identified in cigarette smoke [52].

Group III includes 1-nitropyrene and other 10 compounds. They include structurally unrelated compounds such as furylfuramide, aflatoxin B1 and acridine orange. The previous study indicated that deletion of $umuDC_{ST}$ significantly reduced the mutagenicity of 1-nitropyrene, 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and 3-nitro-6-azabenzo[a]pyrene [34], which are all included in this group. DNA adducts induced by group III compounds appeared to be more efficiently bypassed by DNA polymerase RI leading to −2 frameshift, compared to DNA polymerase IV or DNA polymerase II. Of the compounds, the mutagenicity of 3-nitro-6-azabenzo[a]pyrene is reduced by more than 95% by the deletion of $umuDC_{ST}$ [34]. Since the endogenous DNA polymerase V encoded by $umuDC_{ST}$ is capable to bypass DNA adduct(s) induced by this compound, the exogenous expression of DNA polymerase RI from plasmid pKM101 merely enhanced the mutagenicity by less than three-fold. Although DNA polymerase II encoded by *polB* had virtually no enhancing effects or rather suppressing effects on the mutagenicity of the chemicals examined, the introduction of plasmid pYG787 carrying *polB* specifically and repeatedly enhanced the mutagenicity of benz[a]anthracene, which is a potent carcinogen (Table 3). This compound induces adducts in guanine N^2 and adenine N6 atoms upon metabolic activation [53,54]. Thus, guanine N^2-adducts by the active metabolites of benz[a]anthracene in the CG repetitive sequence could be bypassed by DNA polymerase II leading to −2 frameshift. Since DNA polymerase II is a member of B-family DNA polymerase, its mammalian counterpart such as DNA polymerase delta may have an ability to bypass the adducts in the repetitive sequences.

Group IV includes PBTA-1 and other five compounds. PBTA-1 is a potent aromatic amine mutagen in a polluted river [55]. The characteristic of this group of compounds is that the introduction of any of the plasmids encoding SOS-inducible DNA polymerase had no enhancing effects on the mutagenicity. In the previous study, we reported that the mutagenicity of PBTA-1, Glu-P-1, aminophenylnorharman, N-OH-AAF, 4-NQO and 2-acetylaminofluorene, which are all belong to group IV in this study, are not reduced by the deletions of any of *S. typhimurium* genes encoding SOS-inducible DNA polymerases [34]. Thus, we suggested that the replicative DNA polymerase, i.e., DNA polymerase III holoenzyme, is responsible for the translesion bypass across DNA adducts induced by the chemicals in the CG repetitive sequence leading to −2 frameshift [34]. Although we cannot strictly rule out the possibility that DNA polymerase I is involved in the translesion, we prefer the possibility that DNA polymerase III holoenzyme is responsible for the bypass reactions because the introduction of plasmid pIMA-1 carrying *polA* did not enhance the mutagenicity (Fig. 4). The group IV compounds are all aromatic amines except for 4-NQO and some of them are proved to induce guanine C8 adducts in DNA [56–58]. Thus, we suggest that DNA polymerase III holoenzyme efficiently skips over guanine C8 adducts by aromatic amines in certain sequence context such as CGCGCGCG, thereby inducing −2 frameshift. Involvement of the replicative DNA polymerase may make this repetitive sequence a hot spot for frameshift mutagenesis.

In the previous study, we systematically disrupted one or all the genes of *S. typhimurium* strain TA1538 encoding SOS-inducible DNA polymerases and examined the mutability to chemical mutagens [34]. It is in contrast with the present study where the expression of SOS-inducible DNA polymerases is enhanced. As expected, most of the chemicals exhibited contrastive responses. They displayed enhanced mutagenicity in the presence of enhanced expression of the DNA polymerase, and diminished mutagenicity in the absence of the gene encoding the polymerase. Curiously, some compounds exhibited unexpected mutagenicity in the plasmid-bearing strains and the deletion strains. For example, 3-nitrobenzo[a]pyrene, 1-nitrobenzo[a]pyrene and 2-nitrofluorene were classified into groups I, II and III, respectively (Table 3, Fig. 1). The mutagenicity was enhanced by the introduction of plasmid pYG768 carrying *dinB* encoding DNA polymerase IV and/or pKM101 carrying *mucAB* encoding DNA polymerase RI. However, the mutagenicity of these compounds was not reduced by deletion of any of the genes encoding SOS-inducible DNA polymerases (class IV compounds in the previous study [34]). These results suggest that DNA polymerase III holoenzyme is responsible for the translesion bypass across the lesions induced by the chemicals in physiological conditions, but the Y-family DNA polymerases can take over the translesion reactions when the expression levels are enhanced. In other words, DNA polymerase III holoenzyme, DNA polymerase IV and DNA polymerase RI share, at least in part, the specificity to bypass the lesions and the polymerase actually involved in the translesion depends upon the cellular expression levels or the concentrations of the DNA polymerase in the replication complex.

To examine the possible involvement of DNA polymerase I in the frameshift mutagenesis, we introduced plasmid pIMA-1 carrying *polA* to strain TA1538 and strain YG6215, in which all the genes encoding SOS-inducible DNA polymerase are deleted [34]. Strikingly, the introduction of plasmid pIMA-1 sharply reduced the mutability of strain TA1538 against groups I, II and III compounds to that of strain YG6215 (Fig. 4). The dose–response curves of strain TA1538 harboring plasmid pIMA-1 with benzo[a]pyrene, 10-azabenzo[a]pyrene, 1-aminoanthracene, 3-methylcholanthrene (group I), DMBA, 6-aminochrysene (group II), 1,8-DNP and 1-nitro-6-azabenzo[a]pyrene (group III) almost overlapped those of strain YG6215 harboring plasmid pIMA-1 or the vector plasmid pWKS30. Because the mutability of strain TA1538 harboring plasmid pIMA-1 appears to be similar to that of strain YG6215 harboring the vector, we suggest that the enhanced expression of DNA polymerase I prevents the access of the Y-family DNA polymerases, i.e., DNA polymerase IV and DNA polymerase V, to the replication complex

where translesion bypass actually occurs. It is known that all five DNA polymerases in *E. coli* interact with the beta-subunit of DNA polymerase III holoenzyme [59–61]. Thus, there should be some competition for the polymerases to interact with the beta clamp. The beta-subunit assembles in a donut-like shape as a dimer and tethers DNA polymerase to a template/primer DNA, thereby preventing a falling off of polymerase from template DNA [62]. We speculate the order of the affinity of each DNA polymerase to the beta clamp or the replication complex may be DNA polymerase III > DNA polymerase I > DNA polymerase II > DNA polymerase IV = DNA polymerase V. This assumption is based on the observation that the introduction of plasmid pIMA-1 reduced the mutagenicity of compounds of groups I, II and III, which require the presence of the Y-family DNA polymerases for the maximum mutagenesis, but not group IV, whose mutagenicity is depended upon DNA polymerase III holoenzyme (Fig. 4). In addition, Foster suggested that DNA polymerase II may be dominant over DNA polymerase IV in the replication complex because the *dinB* mutator effects are more pronounced in the stationary-phase mutagenesis when the *polB* gene is deleted [63]. At present, we do not know which of DNA polymerase I or DNA polymerase II has a higher affinity to the replication complex. We prefer the possibility, however, that DNA polymerase I is dominant over DNA polymerase II because it is involved in lagging strand DNA synthesis during the chromosome replication. An alternative explanation for the suppressive effects of plasmid pIMA-1 (Fig. 4) is that DNA polymerase I expressed from the plasmid bypasses the lesions induced by the chemicals of groups I–III in an error-free manner, thereby reducing the mutagenicity. However, we think it less likely because the suppressive effects would vary with chemicals or lesions if DNA polymerase I mediated the error-free TLS. Each DNA polymerase including DNA polymerase I should have specificity to bypass the lesions. Hence, the introduction of pIMA-1 would suppress the mutagenicity of some compounds efficiently but not others. In fact, the strong suppressive effects were observed with almost all the compounds of groups I–III we examined. This is in contrast with the suppressive effects of DNA polymerase II expressed from plasmid pYG787, which reduced the mutagenicity of some of the compounds of groups I–III with various efficiencies (see below more detail). Thus, we prefer the possibility that DNA polymerase I expressed from the plasmid inhibits the access of the Y-family DNA polymerases to the replication complex. Nevertheless, it is important to examine whether a catalytically dead mutant of DNA polymerase I exhibits the suppressive effects on the mutagenicity of groups I–III chemicals to distinguish the possibilities.

In contrast to the clear suppressive effects by plasmid pIMA-1 carrying *polA*, the suppressive effects of plasmid pYG787 carrying *polB* on strain TA1538 were moderate. The introduction of plasmid pYG787 reduced the mutagenicity of benzo[a]pyrene-7,8-dihydroepoxide and 1-aminoanthracene (group I), 6-aminochrysene (group II), 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and furylfuramide (group III) by 40–60% (Table 3). DNA polymerase II may mediate the error-free translesion across DNA adducts induced by these compounds. In the previous study, deletion of $polB_{ST}$ reduced the −2 frameshift mutations induced by benzo[a]pyrene-7,8-tetrahydroepoxide, 3-methycholanthrene, 1-nitropyrene, 1,8-nitropyrene, 1-nitro-6-azabenzo[a]pyrene and 3-nitro-6-azabenzo[a]pyrene by 30–60% [34]. Thus, it seems that the enhanced expression as well as the lack of expression diminished the mutagenicity of 1,8-dinitropyrene and 1-nitro-6-azabenzo[a]pyrene. In other words, DNA polymerase II could have an optimal cellular concentration to enhance the translesion DNA synthesis leading to −2 frameshift. This is contrast to DNA polymerase IV and DNA polymerase RI, which enhance the mutagenesis when the levels of their expression are elevated. *E. coli* DNA polymerase II is reported to be involved in the immediate recovery of DNA synthesis after UV irradiation [13,14]. It is tempting to speculate that it might be required to re-synthesize the primer strand to reach the lesion when the primer strand was degraded. This degradation might occur when DNA polymerase III holoenzyme encountered the lesion and stopped the replication. If the expression level of DNA polymerase II was enhanced, it might promote error-free bypass reactions across the lesions while the lack of DNA polymerase II might lead to poor translesion DNA synthesis by DNA polymerase IV or DNA polymerase V.

In summary, our results suggest that DNA polymerase IV and DNA polymerase RI possess distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, and also that the replicative DNA polymerase, i.e., DNA polymerase III holoenzyme, participates in the bypass reactions in the CG repetitive sequence. Although DNA polymerase III holoenzyme is responsible for the translesion, the Y-family DNA polymerase may take over the primer termini, thereby enhancing the bypass reactions, when the expression of the polymerases is enhanced. Based on the suppressive effects of plasmid pIMA-1, we speculate that the order of DNA polymerases in *E. coli* to access to the replication complex could be DNA polymerase III > DNA polymerase I > DNA polymerase II > DNA polymerase IV = DNA polymerase V. Our results also raise an interesting possibility that strain YG5161 harboring plasmid pYG768 is a sensitive tester strain to identify the mutagenicity of environmental PAHs.

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Regular Article

Development of a Bacterial Hyper-sensitive Tester Strain for Specific Detection of the Genotoxicity of Polycyclic Aromatic Hydrocarbons

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Benzo[a]pyrene (B[a]P), one of polycyclic aromatic hydrocarbons (PAHs), is a ubiquitous environmental pollutant and a potent mutagen and carcinogen. To sensitively detect the genotoxicity of PAHs in complex mixtures extracted from environmental pollutants, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain YG5161 is engineered by introduction of plasmid pYG768 carrying the *dinB* gene encoding *Escherichia coli* DNA polymerase IV into standard Ames tester strain *S. typhimurium* TA1538 (Matsui *et al.*, DNA Repair in press). Strain YG5161 exhibits higher sensitivity to the genotoxicity of B[a]P and other PAHs than do strain TA1538 and TA98. As the conventional Ames tester strains do, however, strain YG5161 also detects the mutagenicity of aromatic amines and nitroaromatics with high sensitivity, which may veil the genotoxicity of PAHs in complex mixtures. *S. typhimurium* possesses strong enzyme activities of nitroreductase and *O*-acetyltransferase, which mediate the metabolic activation of aromatic amines and nitroaromatics and enhance the potent genotoxicity. In this study, we disrupted the *nfsB* and *oat* genes encoding the activation enzymes in strain TA1538 to reduce the cross sensitivity, and introduced plasmid pYG768 into the *ΔnfsBΔoat* strain. The resulting strain YG5185 retained similar high mutability to various chemicals including PAHs as did strain YG5161 and substantially decreased the sensitivity to 1-nitropyrene, 1,8-dnitropyrene and 2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1). We propose that the novel tester strain YG5185 is useful to specifically and sensitively detect the genotoxic PAHs in complex mixtures from various polluted environmental sources.

Key words: genotoxicity, polycyclic aromatic hydrocarbons, complex mixture, *dinB*, translesion DNA synthesis

Introduction

The ambient air and soil of urban centers and other areas can be polluted with potentially carcinogenic and genotoxic chemicals including polycyclic aromatic hydrocarbons (PAHs), most of which are emitted into the atmosphere as a result of incomplete combustion of fossil fuels associated with motor vehicles, industrial activities and home heating (1). In fact, the pollution of air and soil with PAHs is a serious problem in many countries all over the world (2). In Asia, the Chinese government assessed the state of soil contamination on the Beijing outskirts where great changes are undergoing due to the rapid urbanization and industrial development, and concluded that the pyrogenic origins, especially traffic exhausts, are the dominant sources of PAHs (3). In Korea, it is reported that typical soils from agricultural areas contained PAHs at similar level to those in soils from highly industrialized countries (4). In Japan, concentrations of particles of diameter under 1 μm with attached PAHs were measured in various locations in Tokyo and the major polluted places were main traffic roads, highways, and street tunnels (5). In Europe, 20 PAHs and 12 polychlorinated biphenyls (PCBs) in forest soils of Germany were physico-chemically determined, and PAHs were more dominantly detected than PCBs (6). In England, soil samples have been collected from the same plot in 1893, 1944 and 1987 for analysis of PAHs, and it is revealed that the surface soil had been enriched in all PAH compounds, particularly in benzo[a]pyrene (B[a]P) (7). Even in the Southern Hemisphere where pollution levels seem to be lower than those in the northern one, studies of pollution seem to be urgently necessary. In Chile, some persistent toxic substances (PTS) in soils were analyzed, which led to the conclusion that environmental PTS levels are relatively low but PAHs may be of concern in some areas of basin (8). In Brazil, Ames genotoxicity assay was carried out with and without metabolic activation for air samples at four sites in urban area, and higher mutagenic activity was identified at the sites with heavier vehicle traffic. The results using nitroreduc-

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tase- or *O*-acetyltransferase-deficient strains suggest that nitro PAHs seem to be strongly associated with the genotoxicity observed in the urban and industrial regions (9).

To sensitively detect the genotoxicity of B[a]P and other PAHs in complex mixtures extracted from various polluted environmental sources, Matsui *et al* (10). have recently engineered *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain YG5161, which harbored plasmid pYG768 carrying *dinB* encoding *Escherichia coli* DNA polymerase IV in standard Ames tester strain TA1538. The DNA polymerase can bypass a variety of DNA lesions such as those induced by B[a]P (11,12). Thus, the expression of the polymerase enhances B[a]P-induced -2 frameshift events in CGC-GCGCG repetitive sequences in the *hisD* gene in strain TA1538, which reverse the phenotype from His^- to His^+ (13). In fact, the stain exhibits several times higher sensitivity to the genotoxicity of B[a]P than does strain TA1538 or another standard strain TA98 (10). Strain TA98 is the same as strain TA1538 but harbors plasmid pKM101 carrying *mucAB* encoding another Y-family DNA polymerase, i.e., DNA polymerase RI (14). Strain YG5161 also exhibits higher sensitivity to 10-azabenzo[a]pyrene (10-AzaB[a]P), 3-methylcholanthrene (3-MC) and 3-nitrobeno[a]pyrene (3-NB[a]P) than does strain TA1538 or TA98.

Despite the high sensitivity, strain YG5161 possesses a potential problem that is cross sensitivity to genotoxic nitroaromatics and aromatic amines. *S. typhimurium* has strong metabolic activation enzymes for nitroaromatics and aromatic amines, i.e., nitroreductase and *O*-acetyltransferase (15,16). The former is required for the reductive activation of nitroaromatics, and the latter is involved in the activation of *N*-hydroxy compounds derived from nitro- and amino-aromatics. Because of the potent enzyme activities, the genotoxicity of nitroaromatics and aromatic amines is very sensitively detected with *S. typhimurium* tester strains (17). As a consequence, the genotoxic PAHs can be veiled in complex mixtures extracted from various polluted environmental sources if genotoxic nitroaromatics or aromatic amines are contaminated.

In this study, we disrupted the *nfsB* and *oat* genes encoding nitroreductase and *O*-acetyltransferase, respectively, to decrease the cross sensitivity to nitro- and amino-aromatics. Introducing plasmid pYG768 into the *ΔnfsBΔoat* strain resulted in strain YG5185, which retained the high sensitivity to PAHs but exhibited much reduced sensitivity to nitro- and amino-aromatics. We propose that the novel strain YG5185 is useful to detect genotoxic PAHs in the complex mixtures specifically and sensitively.

Materials and Methods

Strains and plasmids: The strains and the plasmids used in this study are listed in Table 1.

Chemicals: The names, CAS registry numbers, abbreviations and sources of the chemicals assayed in this study are as follows: B[a]P (50-32-8), 7,12-dimethylbenz[a]anthracene (57-97-6, DMBA), 2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (67730-11-4, Glu-P-1), and 2-aminoanthracene (613-13-8, 2-AA) from Wako Pure Chemicals (Osaka, Japan); 3-MC (56-49-5) and 1-aminoanthracene (610-49-1, 1-AA) form Sigma-Aldrich Japan K. K. (Tokyo, Japan); 1-nitropyrene (5522-43-0, 1-NP) and 1,8-dinitropyrene (42397-65-9, 1,8-DNP) from Tokyo Kasei Kogyo (Tokyo, Japan). 10-AzaB[a]P (189-92-4) and 1-nitrobenzo[a]pyrene (70021-99-7, 1-NB[a]P) were provided by Drs. Ken-ichi Saeki, Nagoya City University, Nagoya, Japan, and Kiyoshi Fukuhara, National Institute of Health Sciences, Tokyo, Japan, respectively. *N*-Ethyl-*N*′-nitro-N-nitrosoguanidine (4245-77-6, ENNG) is a laboratory stock.

Media: Luria-Bertani broth and agar were used for bacterial culture. Vogel-Bonner minimal agar plates and top agar were prepared as previously described, and used for the His^+ reversion assay with *S. typhimurium* (13). Nutrient broth (Difco, MI, U.S.A.) with ampicillin (AP, 50 μg/ml) was used for pre-cultures of strain YG5161 and YG5185 for the reversion assay.

Construction of nitroreductase deficient strain: Plasmid pYG638 (Fig. 1A) was digested with *Sal*I and *Pvu*II (New England Biolabs, MA, U.S.A.) to remove the replication origin (18), and the 6.8-kb linear *Sal*I-*Sal*I DNA fragment containing the kanamycin-resistance (Km^r) gene between two flanking regions of the *nfsB* gene was purified with JET Sorb extraction kit (Genomed GmbH, Bad. Oeynhausen, Germany) after agarose gel electrophoresis. The purified DNA fragment was treated with T4 DNA ligase (Nippon Gene, Tokyo, Japan), and introduced into *Δoat* derivative of strain TA1538, i.e., strain YG7129, by electroporation (19,20). Colonies resistant to kanamycin were selected, and replacement of the *nfsB* gene with the DNA fragment carrying the Km^r gene was examined by PCR (primers; 5′-TGGAACTGCCTTTTACCGAACACT-3′ and 5′-CCCGGACATAATAGAAAACCGGT-3′) followed by 0.8% agarose gel electrophoresis.

Mutagenicity assay: The mutagenicity assay was carried out with a pre-incubation procedure (13). Briefly, 0.1 mL overnight culture was incubated with the chemicals dissolved in 0.1 mL solvent and 0.5 mL of S9 mix for 20 min at 37°C. When S9 mix is not required, 0.5 mL of 1/15M phosphate buffer pH7.4 was added. The mixture was then poured onto agar plates with soft agar and incubated for 2 days at 37°C. Each chemical was assayed with 4–7 doses on triplicate plates with four

Table 1. Strains and plasmids used in this study

Strain	Genetic characteristic	Source
TA1538	*hisD3052, gal, Δ(chl, uvrB bio) rfa*	(13)
YG5161	the same as TA1538, but harbors pYG768; Ap^r	(10)
YG7129	the same as TA1538, but deficient in *oat*; Cm^r	(19)
YG7158	the same as YG7129, but deficient in *nfsB*; Cm^r Km^r	this study
YG5185	the same as YG7158, but harbors pYG768; Ap^r Cm^r Km^r	this study
plasmid		
pYG638	Derivative of pBR322 for the disruption of the *nfsB* gene; Km^r Ap^r (see Fig. 1)	(18)
pYG768	Derivative of pWSK29 with *dinB* gene; Ap^r	(34)

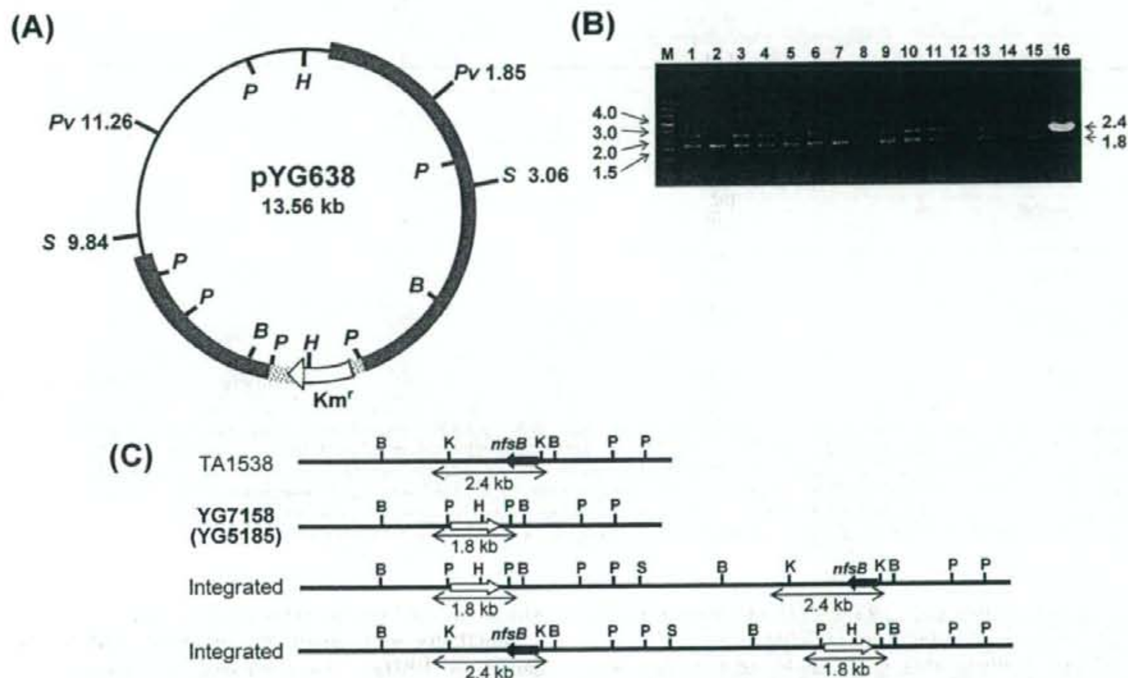

Fig. 1. Disruption of the *nfsB* gene. (A) Physical map of pYG638 (18). The thin and thick gray lines indicate DNA of plasmid pBR322 and the chromosome DNA derived from *S. typhimurium* TA1538, respectively. The dotted region shows the *Pst*I fragment derived from plasmid pUC-4k, which contains the Km^r-gene cassette, whose transcriptional direction is indicated by the arrow head. Symbols for restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I. A *Sal*I-*Sal*I fragment containing the Km^r cassette with the size of 6.78 kb was used for targeted disruption of the *nfsB* gene by pre-ligation method. (B) Result of PCR. PCR products were subjected to 0.8% agarose gel electrophoresis. M indicates size marker and lane numbers are indicated at the top of the gel image. Lanes 2, 5, 7 and 12 indicate the clone whose *nfsB* gene has been replaced with the Km^r gene and lane 16 shows the proper size of the band including the *nfsB*$^+$ gene. Other lanes exhibit the clones that have the *nfsB*$^+$ gene as well as the Km^r-gene fragment integrated into the chromosome. (C) Partial restriction maps of the *nfsB* gene and the surrounding chromosomal region in strain TA1538 and its Km^r recombinants. Closed and open arrows indicate the position and the transcriptional direction of the *nfsB* gene and the Km^r cassette, respectively. Thin arrows indicate the size of the bands amplified by PCR in (B).

strains, i.e., TA1538, YG7158, YG5161, YG5185, in parallel.

Results

Establishment of S. typhimurium strain YG5185: To reduce the cross sensitivity to aromatic amines and nitroaromatics, we disrupted the *nfsB* gene of *S. typhimurium* strain YG7129, which is the same as strain TA1538 but the *oat* gene is already disrupted (Table 1). After electroporation of the DNA fragments containing the Km^r gene instead of the *nfsB* gene into strain YG7129, Km^r colonies were selected. Chromosome

Table 2. Mutagenicity of 11 chemicals in *S. typhimurium* strains with and without plasmid carrying the *dinB* gene encoding *E. coli* DNA pol IV in the presence and the absence of the *nfsB* and *oat* genes

Chemicals	Group	S9	TA1538	YG5161 +DNA pol IV	YG7158 ΔnfsBΔoat	YG5185 ΔnfsBΔoat +DNA pol IV	Dose
10-AzaB[a]P	A	+	2 (1.0)	21 (10.5)	2 (1.0)	28 (14.0)	25 μg/plate
B[a]P	A	+	8 (1.0)	103 (12.9)	9 (1.1)	126 (15.8)	10 μg/plate
3-MC	A	+	8 (1.0)	51 (6.4)	5 (0.6)	48 (6.0)	10 μg/plate
2-AA	A	+	531 (1.0)	1,111 (2.1)	432 (0.8)	1,680 (3.2)	2 μg/plate
DMBA	A	+	5 (1.0)	15 (3.0)	4 (0.8)	9 (1.8)	5 μg/plate
ENNG	A	−	2 (1.0)	11 (5.5)	2 (1.0)	12 (6.0)	10 μg/plate
1-AA	B	+	18 (1.0)	53 (2.9)	5 (0.3)	14 (0.8)	10 μg/plate
1-NB[a]P	B	−	192 (1.0)	2,042 (10.6)	72 (0.4)	326 (1.7)	0.5 μg/plate
1-NP	C	−	145 (1.0)	230 (1.6)	23 (0.2)	29 (0.2)	1 μg/plate
1,8-DNP	C	−	130,560 (1.0)	218,040 (1.7)	2,040 (0.02)	3,120 (0.02)	25 ng/plate
Glu-P-1	C	+	41,240 (1.0)	45,550 (1.1)	1,710 (0.04)	1,670 (0.04)	100 ng/plate

Each chemical was assayed with 4–7 doses on triplicate plates with four strains in parallel. The numbers of His$^+$ revertants per plate per μg are calculated at the doses indicated with arrows in Fig. 2. The numbers in parenthesis represent the values relative to the numbers of His$^+$ revertants per μg in TA1538. Differences of relative mutability more than two fold or less than half were regarded as significant effects of the introduction of plasmid pYG768 carrying *dinB* encoding DNA pol IV or the deletion of the *oat* and *nfsB* genes on the mutability. Group A: chemicals whose mutagenicity was enhanced by DNA pol IV but was not reduced by Δ*nfs*Δ*oat*; Group B: chemicals whose mutagenicity was enhanced by DNA pol IV and was reduced by Δ*nfs*Δ*oat*; Group C: chemicals whose mutagenicity was not enhanced by DNA pol IV but was reduced by Δ*nfs*Δ*oat*.

DNA surrounding the *nfsB* gene was amplified from the colonies by PCR using primers flanking the *nfsB* gene, and the products were analyzed by agarose gel electrophoresis (Fig. 1B). If the chromosomal *nfsB* gene is correctly replaced with the introduced DNA fragments carrying the Km^r gene by recombination using the flanking homologous sequences, the Km^r colonies will exhibit single DNA bands of 1.8 kb. If no such true replacement occurs and the introduced DNA fragments are only integrated into the vicinity of the chromosomal *nfsB* gene, they will exhibit double bands of 2.4 kb (the intact *nfsB* gene) and 1.8 kb (the Km^r gene). As expected, some Km^r colonies exhibited single 1.8-kb bands while others exhibited double bands of 2.4 kb and 1.8 kb. We suggested that the Km^r colonies exhibited single 1.8-kb bands were deficient in the *nfsB* gene as well as the *oat* genes, and named the resulting strain YG7158. Plasmid pYG768 carrying the *dinB* gene encoding *E. coli* DNA polymerase IV was introduced and the resulting strain was referred to as YG5185, which was used for the subsequent mutation assays.

Specificity and sensitivity of strain YG5185 to genotoxic PAHs: We compared the sensitivity of strain YG5185 to 11 mutagens with those of three reference strains: the parent strain, i.e., TA1538, the derivative of strain TA1538 harboring plasmid pYG768, i.e., YG5161, and the Δ*nfsB*Δ*oat* derivative of strain TA1538, i.e., YG7158 (Table 2). Introduction of plasmid pYG768 did not affect the spontaneous mutation frequencies in strains TA1538 and YG7158. Based on the order of sensitivity of the strains, we classified the chemicals into Group A to C as follows. For Group A compounds, i.e., 10-AzaB[a]P, B[a]P, 3-MC, 2-AA, DMBA and ENNG, deletion of the *nfsB* and *oat* genes did not reduce the sensitivity, and introduction of plasmid pYG768 substantially enhanced it (Fig. 2A). Thus, YG5185 exhibited similar or comparable sensitivity to Group A compounds as did strain YG5161, and the order of the sensitivity was YG5185 = YG5161 > TA1538 = YG7158. For Group B

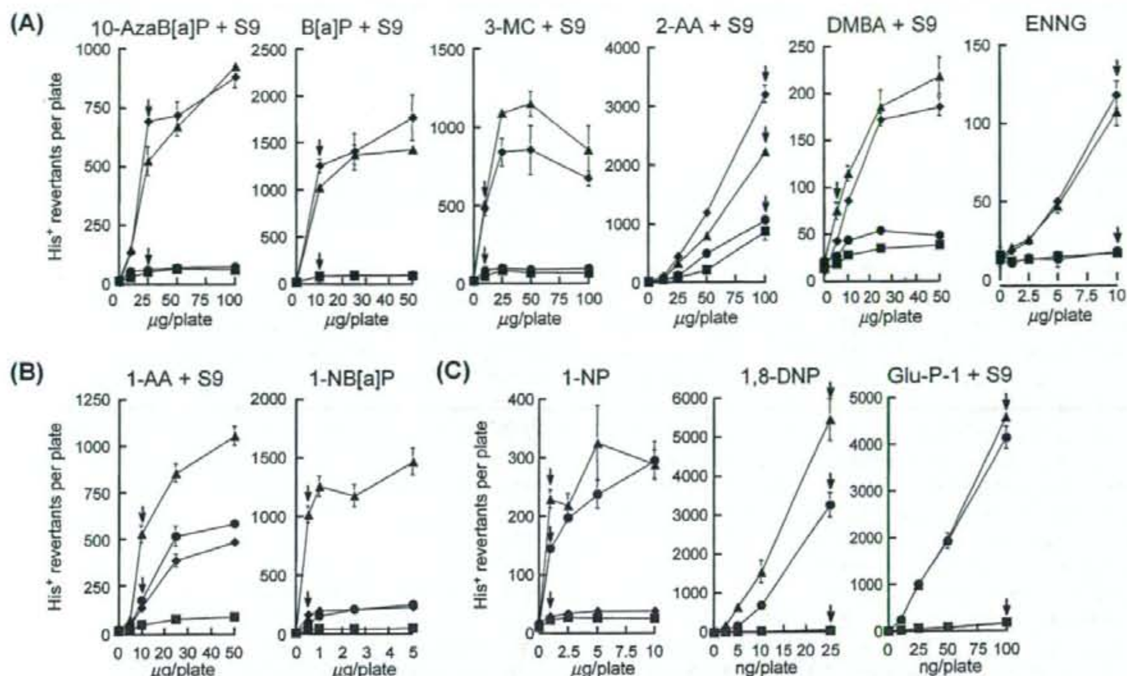

Fig. 2. Mutagenic responses of *S. typhimurium* newly constructed strains in the Ames test. The test chemicals are indicated on each panel. The chemicals are categorized into three Groups A, B and C, which are mentioned in the text and Table 2. Symbols are: ●, TA1538; ▲, YG5161; ■, YG7158; ◆, YG5185. The arrow indicates the dose that was used for the calculation of His$^+$ revertants per μg per plate in Table 2.

compounds, i.e., 1-AA and 1-NB[a]P, deletion of the *nfsB* and *oat* genes substantially reduced the sensitivity, and introduction of plasmid pYG768 enhanced it (Fig. 2B). Thus, the order of the sensitivity was YG5161 > TA1538 > YG5185 > YG7158. For Group C compounds, i.e., 1-NP, 1,8-DNP and Glu-P-1, deletion of the *nfsB* and *oat* genes severely reduced the sensitivity, and introduction of plasmid pYG768 did not substantially enhance it (Fig. 2C). The order of the sensitivity was YG5161 = TA1538 > YG5185 = YG7158.

Discussion

Genetically engineered Ames tester strains have been proven to be useful in environmental genotoxicology due to their extreme sensitivity and the mechanistic information they can provide (17). For example, *S. typhimurium* strain YG1021, 1024, 1026 and 1029 have been widely used for environmental research for their hypersensitivity to the genotoxic action of nitroarenes and aromatic amines (21). The strains harbor plasmids carrying *nfsB* or *oat*, conferring high enzymatic activities of nitroreductase or *O*-acetyltransferase in strain TA98 or TA100 (15,16,22,23). Strains YG7104 and YG7108, whose repair systems for the damage by alkylating agents are disrupted, exhibit hyper sensitivity to alkylating agents, such as methyl methanesulfonate and dimethylnitrosamine (24,25), and they are used for mechanistic analyses for chemical mutagenesis and carcinogenesis (26–28). Strain YG3001, YG3002 and YG3003 are highly sensitive to oxidative mutagens due to the lack of the *mutM* gene encoding 8-hydroxyguanine DNA glycosylase (29), and are shown to be useful for the studies on oxidative DNA damage (30,31).

To expand this line of research, we have previously established *S. typhimurium* strain YG5161 by introduction of plasmid carrying *dinB* encoding *E. coli* DNA polymerase IV into strain TA1538 to increase the sensitivity to genotoxic PAHs (10). Because of the error-prone nature of DNA polymerase IV, strain YG5161 exhibited higher sensitivity to PAHs such as B[a]P, 10-AzaB[a]P and 3-NB[a]P than did standard Ames strain TA1538 or TA98. Nevertheless, strain YG5161 has a potential defect as a bio-detector of genotoxic PAHs in complex mixtures, which is the cross sensitivity to other classes of genotoxic compounds, i.e., nitroaromatics and aromatic amines. Since these compounds are ubiquitously present in the environment and their genotoxicity in *S. typhimurium* is extremely amplified by the presence of intracellular metabolic activation enzymes, i.e., nitroreductase and *O*-acetyltran-

sferase, they can veil the potential genotoxicity of PAHs in the complex mixtures (17). For example, 1,8-DNP could be dominantly detected as a principle genotoxic compound in complex mixtures if 1,8-DNP and B[a]P were present at a weight ratio of 1:1,000. This is because the genotoxicity of 1,8-DNP, i.e., the numbers of His^+ revertants per plate per μg, is more than 15,000 times higher than B[a]P in strain TA1538 (Table 2). Despite the potent genotoxicity in *S. typhimurium*, 1,8-DNP is categorized into Group 2B (possible human carcinogens) by International Agency for Research on Cancer (IARC) while B[a]P is classified into Group 2A (probable human carcinogens) (32). Hence, we found it important to increase the specificity of tester strains to genotoxic PAHs.

Here, we disrupted the *nfsB* and *oat* genes of strain TA1538, introduced plasmid pYG768 carrying *dinB* into the *ΔnfsBΔoat* strain and established novel *S. typhimurium* strain YG5185 (Table 1). When compared the sensitivity of strain YG5185 and YG5161, they exhibited comparative sensitivity to Group A compounds where four out of six compounds, i.e., 10-azaB[a]P, B[a]P, 3-MC and DMBA, were PAHs (Table 2, Fig. 2A). Thus, strain YG5185 appears to be able to detect the genotoxic PAHs with similar high sensitivity as does strain YG5161. The remaining two compounds in Group A are 2-AA and ENNG. It cautions that the compounds that are more sensitively detected by strain YG5161 or YG5185 compared with strain TA1538 are not necessarily PAHs. They can be aromatic amines or alkylating agents.

For Group B compounds, i.e., 1-AA and 1-NB[a]P, the genotoxicity was significantly reduced by the deletion of *nfsB* and *oat*, as in the case of Group C compounds (Fig. 2B and C). Unlike Group C compounds, however, the genotoxicity of 1-AA and 1-NB[a]P was three to 10 times enhanced by the introduction of plasmid carrying *dinB* (Fig. 2B). Actually, the genotoxicity of two compounds was more sensitively detected with strain YG5161 compared with standard strain TA98 (10). Thus, it seems that DNA lesions induced by 1-AA and 1-NB[a]P are more efficiently bypassed by DNA polymerase IV in an error-prone manner than by DNA polymerase RI encoded by *mucAB* carried by plasmid pKM101 in strain TA98. Interestingly, the genotoxicity of 1-AA was reduced by more than 70% by the deletion of *nfsB* and *oat* while the genotoxicity of 2-AA was not (Table 2, Fig. 2A and B). These results suggest that the intracellular metabolic activation mechanisms are markedly different between two aromatic amino compounds despite the structural similarity.

In Group C compounds, the deletion of *nfsB* and *oat* reduced the genotoxicity of 1-NP by more than 85% and those of 1,8-DNP and Glu-P-1 by more than 95% (Table 2). Strain YG5185 exhibited much lower sensitivity to the genotoxicity of nitroaromatics and aromatic amine than did strain YG5161 (Fig. 2C). Thus, we concluded that strain YG5185 more specifically detects the genotoxicity of PAHs than does strain YG5161. Strain YG5185 could help and facilitate the successful isolation of genotoxic PAHs in complex mixtures. We have to point out, however, that the genotoxicity of 1,8-DNP and Glu-P-1 in strain YG5185 is still more than 25 times and 10 times higher than that of B[a]P. Hence, there is a possibility that genotoxic nitroaromatics or aromatic amines can be detected as principle mutagens if the complex mixtures are heavily contaminated with nitroaromatics or aromatic amines.

During the strain construction, we noticed that *S. typhimurium* TA1538/1,8-DNP could have mutations in the genes other than the *oat* gene. This is because introduction of plasmid pYG768 carrying *dinB* did not enhance the sensitivity of the strain to B[a]P while introduction of the same plasmid into YG7158 (*ΔnfsBΔoat* strain) or YG7129 (*Δoat* strain) enhanced it more than 10 times. It may not be surprising that strain TA1538/1,8-DNP has unexpected mutations because it was generated by random mutagenesis with 1,8-DNP (33). To avoid such confusion by extra mutations, we specifically disrupted the *oat* and *nfsB* genes by a targeting method, i.e., the pre-ligation method, which has been developed in this laboratory (20).

In summary, we established novel *S. typhimurium* strain YG5185 by introduction of plasmid carrying *dinB* encoding *E. coli* DNA polymerase IV into *ΔnfsBΔoat* derivative of standard Ames tester strain TA1538. The newly constructed strain exhibited higher sensitivity to the genotoxic compounds including PAHs but reduced sensitivity to nitroaromatics and aromatic amines. We propose that strain YG5185 is useful to detect the genotoxic PAHs in complex mixtures extracted from various polluted environmental sources.

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