APPENDICES

Appendix 1 Formulae and physical properties of 16 EPA-PAHs.

Compound	Formula	Molecular weight	Melting point (°C)	Boiling point (°C)	CAS No.*
ACE	$C_{12}H_{10}$	154.2	96.2	279	83-32-9
ACY	$C_{12}H_8$	152.2	92-93	265-275	208-96-8
ANT	$C_{14}H_{10}$	178.22	218	342	120-12-7
BaA	$C_{18}H_{12}$	228.3	158-159	-	56-55-3
BaP	$C_{20}H_{12}$	252.3	177	310-312	50-32-8
BbF	$C_{20}H_{12}$	252.3	168	-	205-99-2
BPE	$C_{22}H_{12}$	276.3	273	-	191-24-2
BkF	$C_{20}H_{12}$	252.3	217	480	207-08-9
CHR	$C_{18}H_{12}$	228.3	255-256	-	218-01-9
DBA	$C_{22}H_{14}$	278.4	262	-	53-70-3
FLA	$C_{16}H_{10}$	202.3	110	-	206-44-0
FLU	$C_{13}H_{10}$	166.2	116-117	293-295	86-73-7
IND	$C_{22}H_{12}$	376.3	161.5-163	-	193-39-5
NAP	$C_{10}H_8$	128.2	80.2	217.9	91-20-3
PHE	$C_{14}H_{10}$	178.2	100	340	85-01-8
PYR	$C_{16}H_{10}$	202.3	156	399	129-00-0

* Chemical Abstracts Service Registry Number.

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Appendix 2 IARC classification of chemical carcinogenicity to humans.

Category	Definition
Group 1	The agent is carcinogenic to humans
Group 2	
Group 2A	The agent is probably carcinogenic to humans
Group 2B	The agent is possibly carcinogenic to humans
Group 3	The agent is not classifiable as to its carcinogenicity to humans
Group 4	The agent is probably not carcinogenic to humans

Appendix 3. Procedures for confirmation of genotypes of tester strains

3.1 The histidine requirement

The his- character of the tester strains was confirmed by demonstrating the histidine requirement for growth on selective agar plates. Biotin was also required by all of the standard tester strains(except TA102) because of the uvrB deletion which extends through the Bio gene.

Procedure: One-tenth ml of o.1 M L-histidine was spread thoroughly to the surface of minimal glucose agar plate after histidine was completely taken up by the agar, 0.1 ml of 0.5 mM biotin was spread thoroughly again. Control plates were spread with biotin only but no histidine. After the two solutions were completely taken up by agar, the sterile wire loop was dipped into the culture and was made a single sweep across the biotin control plate and then across the histidine/biotin plate. The plates were incubated overnight at 37°C. The histidine tester strains were grown on only the histidine/biotin plates. On the control plates there should be no growth of bacteria.

3.2 The crystal violet sensitivity test (to confirm rfa mutation)

Procedure: Two ml of molten top agar which has held at 45°C was added to a sterile tube containing 0.1 ml of 10 mM L-histidine, 0.1 ml of 1 mM biotin, and poured on a nutrient agar plate. The plate was tilted and rotated to distribute the top agar, then was placed on a level surface. After the agar became firm, a sterile crystal violet filter paper disc was pressed on the seeded plates. After 12-24 hours incubation at 37°C, a clear zone of inhibition (about 14 mm, diameter) around the disc was seen, indicating the presence of the rfa mutation.

3.3 The ampicillin resistant test (to confirm the R-factor strains)

The ampicillin resistance factor was tested because the plasmid was somewhat unstable and can be lost from the bacteria.

Procedure: A sterile filter paper disc containing ampicillin (10 μl of 8 mg/ml in 0.02 N NaOH) was placed on the nutrient agar plates seeded with 0.1 ml of 10 mM L-histidine, 0.1 ml of mM biotin and 0.1 ml of fresh overnight culture bacteria. After incubation for 12-24 hours at 37°C, the indication of ampicillin resistance was shown by absence of zones of inhibition around the discs.

3.4 The uvrB mutation

The presence of uvrB mutation was confirmed by testing UV sensitivity.

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Procedure: The tester culture was streaked across a nutrient agar plate, in parallel stripes. Half of the plate was irradiated with long wavelength UV lamp (366 nm) at the distance of 33 cm for 8 seconds, so that half of each bacterial streak was unirradiated. The plate was incubated at 37°C for 12-24 hours. The bacteria with uvrB mutation should grow only on the unirradiated side of the plate.

3.5 Spontaneous mutation

The spontaneous reversion of the tester strains is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. The revertant per plate without metabolic activation was 20-50 for TA98, 100-200 for TA100; the number may be slightly different on plates with metabolic activation (S9).

Procedure: One-tenth ml of fresh overnight culture of bacteria was mixed with 50 μl DMSO with and without S9 and preincubated at 30°C for 30 minutes. After preincubation 2.0 ml of molten top agar at 45°C was added into the mixture and poured on the minimal glucose agar plate. The plates were incubated at 37°C for 48 hours and the spontaneous revertant colonies were scored.

3.6 Positive controls

The positive mutagenesis controls using diagnostic mutagens to confirm the reversion properties and specificity of each strain and the efficiency of the S9 mix. In this study 2AA and AF-2 were used as diagnostic mutagens for metabolic activation and without metabolic activation, respectively.

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Appendix 4 Preparation of some reagents

4.1 Preparation of minimal glucose agar plate

The components of 1,000 ml minimal glucose agar medium were

Bacto-Difco agar	15 g
Distilled water	850 ml
10 x Vogel-Bonner medium E	100 ml
40 % Glucose	50 ml

The ingredients should be autoclaved separately, when the solution has cooled slightly, added together, mixed well and poured 30 ml into each plate.

The components of 1,000 ml the Vogel-Bonner medium E (ten-fold solution) are

$MgSO_4.7H_2O$	2 g
Citric acid. H_2O	20 g
K_2HPO_4	100 g
$NaNH_4HPO_4.4H_2O$	35 g

4.2 Preparation of top agar containing histidine and biotin

A: The components of 10 ml top agar

Bacto-Difco agar	0.6 g
NaCl	0.5 g
Distilled water	100 ml

The solution was sterilized by autoclave at 1 lb, 120°C, 20 min.

B: The components of 100ml 0.5 mM histidine/biotin

Ingredient	per liter
D-Biotin	124 mg
L-Histidine HCl H_2O	105 mg
Distilled H_2O	100 ml

Dissolve histidine and biotin by stirring in water. Sterilize by filtration through membrane filter (0.22 μm pore size). Depending on number of plates for each test, calculate a total volume of top-agar required (according to the amount of top agar used = 2 ml per plate). The Histidine/biotin solution will be added to the top agar before starting the mutagenic experiment by the ratio of 10 ml of solution B : 100 ml solution A.

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