CHAPTER-II : GENERAL MATERIALS AND METHODS
<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Relevant genetic markers</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>TA97a</td>
<td>uvrB, hisD6610, bio, rfa, R-factor plasmid-pKM101</td>
<td>Ames, B.N.</td>
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<td>TA98</td>
<td>uvrB, hisD3052, bio, rfa, R-factor plasmid-pKM101</td>
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<td>TA100</td>
<td>uvrB, hisG46, bio, rfa, R-factor plasmid-pKM101</td>
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<td>TA102</td>
<td>rfa, R-factor plasmid-pKM101, multicopy plasmid-PAQ1 containing hisG428 auxotrophic markers and tet^</td>
<td>Ames, B.N.</td>
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<td>TA104</td>
<td>uvrB, hisG428, rfa, R-factor plasmid-pKM101</td>
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<td><strong>E. coli K-12 Strains</strong></td>
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<td>AB1157</td>
<td>thi-1, argE3, thr-1, leuB6, proA2, hisG4, lacY1, F^, Str^, s</td>
<td>Howard-Flanders, P.</td>
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<td>AB2463</td>
<td>recA13, thi-1, argE3, thr-1, leuB6, proA2, hisG4, F^, Str^, s</td>
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<td>AB2494</td>
<td>lexA, thi-1, thr-1, leuB6, proA2, hisG4, metB, lacY1, F^, Str^, s</td>
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<td>KL400</td>
<td>thi-1, lex66, proC32, hisF860, lac736, molA38, ara-14, mtl-1, xyl-5, strA109, spe-15.</td>
<td>Barbara, J.B.</td>
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<td>KL403</td>
<td>polA1, ara-14, lex86, lac236, proC32, hisF860, thyA54, rpsE2115, rps2109, molA38, xyl-5, mtl-1, thi-1</td>
<td>Barbara, J.B.</td>
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Methods

Sample collection and situation of the collection sites

The Ganga River is the largest river in India covering a distance of about 2,525 km in the states of Uttar Pradesh, Bihar and West Bengal. The present study is concerned with about 250 km stretch of the river from Narora to Kannauj in Uttar Pradesh. A total of 48 water samples were collected for a period of one year between March 1989 to March 1990 usually at 30 days interval viz. from four sampling stations i.e. Narora, Kachhla, Fatehgarh and Kannauj. Narora was located at a latitude 20.10′,30″ and longitude 78.24′15″, Kachhla’s location was at a latitude27.55′58″ and longitude 78.15′,29″. Fatehgarh at latitude 27.24′7″ and longitude 79.37′55″ and Kannauj at latitude 27.0′,3″ and longitude 79.59′18″. Samples were collected in sterile glass bottles about 30 cm under the water surface. The time between sample collection and extraction did not exceed 6 hr. The liquid extracts and XAD concentrates were, however, stored at -20°C for longer times ranging from 1 week to 16 months.

Preparation of concentrated water extracts

(1) XAD-Extraction method: For the concentration of organic constituents a volume of 16 to 20 liter of river water was collected per sample. The samples were collected from the four stations of the upper middle stretch of Ganga river viz. Narora, Kachhla, Fatehgarh and Kannauj. Before concentration, the water samples were filtered over two membrane filters with pore size 8μm and 0.45μm (Millipore
waters). Adsorption of the organic constituents on the XAD resins were carried out as described by Wilcox and Williamson (1988). XAD-4 and XAD-8 resins were obtained from Serva GmbH, Heidelberg, F.R. Germany. For about a 1000-fold concentration, 20 liters of filtered river water were passed through the washed column at a constant temperature of 20-25°C and with a flow rate of 5-20 ml/min. The adsorbed organic material was then eluted with about 20 ml of acetone. This eluate was evaporated to dryness and reconstituted in DMSO such that 1 ml of extract was equivalent to 10 liter of original water. These samples were filter-sterilized through 0.45μm membrane filters and stored at -20°C until testing was completed.

(ii) Liquid-Liquid Extraction: Organochlorine and organophosphorus pesticides were isolated by liquid-liquid extraction method as described by Singh et al. (1987). About one liter of the well mixed water samples were extracted 3 times with 50 ml n-hexane (HPLC-grade) by continuous shaking. About 150 ml hexane extract contained all the organochloride pesticides. Organophosphorus pesticides were extracted from the same water sample which was already extracted with hexane. In this water sample, 50 ml chloroform (HPLC-grade) was added 3 times and 150 ml chloroform extract was collected which contained organophosphorus pesticides. Extracted organic phases were transferred to the round bottom flask.
and evaporated at 40°C under reduced pressure with the help of vacuum pump and reduced the volume of extract to 2 ml. The extract was then transferred into a 5 ml volumetric flask and made up the volume up to the mark. For mutagenicity testing about 10 liters of river water was extracted, and the volume was reduced to 1 ml. These samples were filtered through 0.45μm membrane filter before they were used for pesticide analysis in HPLC and mutagenicity testing.

Maintenance and growth of bacteria: Each strain of Salmonella typhimurium was streaked over master plate. A single colony was picked up, grown in minimal medium and repurified by streaking over fresh master plate. Likewise each strain of E. coli was purified by streaked over nutrient agar plates. The cultures were regularly tested on the basis of associated genetic markers raising them from a single colony from the master plates. Having satisfied with the test clone the culture was raised and streaked over minimal and nutrient agar slants. It was then allowed to grow 0/N at 37°C and stored at 4°C. Every month cultures were transferred over fresh slants with TA102 strain as an exception. It was transferred after every 15 days. Stabs were also prepared for longer storage.

Over night culture of S. typhimurium strains were used as such for experiments. Overnight culture of E. coli were raised in nutrient broth at 37°C. The culture was diluted fifty times till the cell density reached to about 2 x 10^8 viable counts ml⁻¹. Such exponential cultures were used in all the experiments.
Materials

Media for Ames Strains

Medium for master plates and slants: The composition of the medium for Ames strains to prepare master plates and slants was as under:

- Sterile 50 x VB Salts* 20 ml
- Sterile agar 15g/910 ml
- Sterile 40% glucose 50 ml
- Sterile histidine HCl.H2O 10 ml
  (2g per 400 ml H2O)
- Sterile 0.5 mM biotin 6 ml
- Sterile ampicillin solution 3.15 ml
  (8mg/ml 0.02M NaOH)
- Sterile tetracycline solution**
  (8mg/ml 0.02 N HCl)

For the preparation of plates, the above components were mixed with the molten agar.

**Tetracycline was added only for use with TA102 which is tetracycline resistant.

*Stock solution of VB salts (IX) was prepared using the following ingredients:

- MgSO4.7H2O 0.2 g/l
- Citric and monohydrate 2.0 g/l
- K2HPO4 (anhydrous) 10.0 g/l
- Na2NH4PO4.4H2O 3.4 g/l

The salts were added in the order indicated to warm distilled water and each salt was allowed to dissolve completely before adding the next. The solution was then
autoclaved for 20 min at 121°C.

**Minimal glucose plates for mutagenicity assay:**

- Sterile VB Salts : 20 ml
- Sterile 40% glucose : 50 ml
- Sterile agar : 15 g/930 ml distilled water

The above components were mixed with the molten agar and then 30 ml was poured over each plate.

**Top agar for mutagenicity assay:** The top agar contained 0.6% agar powder and 0.5% NaCl. 10 ml of sterile solution of 0.5mM histidine. HCl/0.5mM biotin was added to the molten agar and mixed thoroughly by swirling.

**0.5mM histidine/biotin solution for mutagenicity assay:**

- D-Biotin : 30.0 mg
- L-Histidine HCl : 24.0 mg
- Distilled water : 250.0 ml

First biotin was dissolved by heating the water to the boiling point. Then histidine was mixed to it and finally the solution was sterilized for 20 min at 121°C.

**0.2 M Sodium phosphate buffer, pH 7.4:**

- \( \text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \) : 13.8 g/500ml
- \( \text{Na}_2\text{HPo}_4 \) : 14.2 g/500ml

The pH was adjusted to 7.4 and sterilized at 121°C for 20 min.
Sg mix for mutagenicity assay: Sg (Livers of Sprague-Dawley rats, Aroclor 1254-induced) mix was prepared by mixing (per 3.0 ml) sterile distilled water, 1.185 ml; 0.2M NADP, 0.120 ml; 1M glucose-6-phosphate, 0.015 ml; sterile 0.4M MgCl₂-1.65M KCl salts, 0.060 ml; Rat Liver Sg fraction, 0.120 ml (4%). Sg mix was freshly prepared for each experiment.

Media for E.coli K-12 strains

Nutrient broth (13 g/l): Nutrient broth obtained from Hi-media (India) had the following composition:

- Teptone 5.0 g/l
- NaCl 5.0 g/l
- Beef extract 1.5 g/l
- Yeast extract 1.5 g/l
- pH (approx.) 7.4 0.2

In the nutrient broth obtained from Difco (U.S.A.), NaCl was also added to it —

- Nutrient broth 8 g/l
- NaCl 5 g/l

Nutrient agar (Hard agar):

- Nutrient broth 13 g/l
- Agar powder 15 g/l

MgSO₄·7H₂O Solution (0.01M): For all dilutions, 0.01 M MgSO₄ solution was used.
Buffers and solutions for in vitro tests

0.01M TNE buffer: For preparing DNA solution. A 2 mg/ml solution of calf-thymus DNA was prepared in 0.01M TNE or TN (0.01M Tris-HCl, pH 7.5; 0.01M NaCl and 10⁻⁴ M EDTA) Buffer.

1M Sodium phosphate buffer (pH 7.0): A 1 M sodium phosphate buffer was prepared for hydroxyapatite chromatography. Further dilutions of the buffer were done with this stock buffer.

Solutions for S₁ nuclease hydrolysis

S₁ nuclease dilution (1 unit/ul):

- Sterilized S₁ nuclease buffer (pH 4.5) 200 ul
- Sterilized glycerol (5%) 50 ul
- Sterilized distilled water 744 ul
- S₁ nuclease 6 ul (=1000 units)

S₁ nuclease buffer pH 4.5:

- 0.5M sodium acetate (pH 4.5)
- 1.0mM ZnSO₄

Perchloric acid was used at strength of 14% and bovine serum albumin 10 mg/ml in sterilized distilled water.

Diphenylamine reagent for the estimation of DNA:

- Diphenylamine 250 mg
- CH₃COOH (glacial) 25 ml
- H₂SO₄ (conc.) 675 ul
In Vitro Testing of Sister Chromatid Exchange (SCE)

Preparation of culture media: Tissue culture 199 with α-glutamine and Hapes buffer without NaHCO₃ was always prepared in advance and stored at 4°C, but the storage never tasted longer than a week. 1.5ug of medium was dissolved in 100 ml of triple distilled water by gentle shaking. Then, penicillin (100 u/ml) and streptomycin (100 v/ml) were added and the pH was adjusted to 6.8 - 7.2 with NaHCO₃ or HCl. The medium was sterilized by using the 0.45um millipore filters. The filtered medium was then stored in sterilized, tightly capped glass bottles.

Collection of blood Samples: Peripheral blood from the healthy donor was taken (fresh every time) through vein puncture under aseptic conditions using disposable needles (21 guage) and disposable syringes. Heparin (1000 u/ml) was used as anticoagulant. The samples of blood were immediately transferred to heparinized vials.

Setting up the cultures: Lymphocyte culture was prepared by adding 0.2ml of blood to 3 ml of culture medium supplemented with 0.1ml phytohemagglutinin (PHA-P) and 15% Fetal Calf Serum. The culture vials were then tightly capped to avoid CO₂ loss and after gently mixing their contents these were incubated at 37°C in dark for 72 hrs. Colchicine (0.20ug/ml) was added 1/2 hrs prior to harvesting, to arrest the cells at metaphase stage.

Harvesting of the Culture: After the 72 hrs incubation, the cultures were taken out from the incubation and were
transferred to centrifuge tube. The cells were spun down by centrifugation (1000 rpm) and the supernatant were discarded.

Hypotonic treatment (0.075M KCl) were given for 15 min at 37°C and cells recollected by centrifugation. After that the cells were suspended in 8ml freshly prepared chilled fixative. Methanol: acetic acid (3:1) was added drop by drop with a pasteur pipette while continuously shaking the pellet as to avoid the formation of clots. In order to ensure the proper fixation, the cells were kept suspended in the fixative for a period of overnight preferably. Two or three changes with fresh fixative were given before preparing the slides.

**Slide preparation:** After giving final washing in the fixative, the cells were resuspended in 5ml of fresh fixative. Two or three drops of cell suspension were dropped on dry, clean, grease free, pre-chilled net microscope slides.