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## **Salmonella as a Unique Tool for Genetic Toxicology**

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### **1. Introduction**

In molecular biology and genetics, mutations are described as sudden and spontaneous or induced changes in a genomic sequence (Brown, 2007). They have wide effects on all living organisms from bacteria with a single prokaryotic cell construction to multicellular and eukaryotic organisms including human being with high-level cellular differentiation. Mutations occur also in the genomic materials (DNA or RNA) of viruses and affect their functionality (Hartl & Jones, 1998; Lewin, 2004). When a mutation happens, it can basically result in several different types of change in DNA (or RNA for some viruses) sequences; these can have no effect, alter the product of gene, and prevent the gene from functioning properly or completely. Alterations in the product of gene and partial or total loss of gene function generally result in a disadvantageous situation for the organism, which cause various symptoms and ailments affect the maintenance of life (Brown, 2007). Previous studies made to understand the relations between mutations and their negative effects on human being clearly showed that some diseases, such as most forms of cancer, heart disease and mental disorders, have a partly or completely genetic basis closely related to mutagenesis (Bertram, 2000; Alberts et al., 2002; Lodish et al., 2007). Therefore, recent investigations have mainly focused on mutation classification, understanding mutagenesis mechanisms, determination of mutagenic agents and prevention strategies (Cox, 1976; Albertini et al., 1990; Davidson et al., 2002; Akiyama, 2010; Evans et al., 2010; Gulluce et al., 2010; Lynch, 2010; Waters et al., 2010; Lange et al., 2011; Loeb, 2011; Pao & Girard, 2011). Thus, the identification of substances capable of inducing mutations has become an important procedure in safety assessment. In the research studies, mutations can be divided in two main groups to get more comprehensive results according to their size. First group is described as gene mutations, where only single base is modified, or one or a relatively few bases are inserted or deleted (Brown, 2007). Other one consists of chromosome mutations, which are including chromosome breaks, large deletions, rearrangements, or gain or loss of whole chromosome (Hartl & Jones, 1998; Lewin, 2004).

Mutation test systems also divide in long-term and short-term systems according to obtaining of the results (Wickramasnghe, 1979; Mortelmans & Zeiger, 2000; Zeiger et al., 2005). The long-term mutagenicity tests, which use *in vivo* researches with various experimental animals, give more reliable results than short-term mutagenicity test systems. However, they are not preferred as beginning test systems due to their high cost and long time requirements, where mutagenic potential of many synthetic and natural chemicals are checked (Wickramasnghe, 1979; Gulluce et al., 2010). In these studies, the short-term test

systems, which eliminate disadvantages of the long-term test systems, are more suitable and acceptable. Many short-term studies result in gaining reliable and alternative data under controlled *in vitro* conditions. Another important advantage is that short-term mutation test systems are not only correlated with other short-term test systems, but also long-term systems (Mortelmans & Zeiger, 2000). Thus, the combinations of the mutagenicity test systems play a key role to get more meaningful results.

The bacterial mutation assays are known as most important short-term systems in order to determine mutagenic and antimutagenic potential of natural or synthetic chemicals related to gene mutations (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Mortelmans & Riccio, 2000; Mortelmans & Zeiger, 2000). The *Salmonella* bacterial reverse mutation assay is one of the simplest, the most meaningful and acceptable short-term mutagenicity and antimutagenicity test systems. The test was initially developed by Ames in 1971. Therefore, it is also called as the Ames mutagenicity assay or the Ames/*Salmonella* mutagenicity assay (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Zeiger, 2004, 2010). The main advantages of the assay, which employs mutant *Salmonella typhimurium* tester strains as model prokaryotic organisms, can be ordered as inexpensive applications enable studying a large number of test materials, quickly resulting (approximately 48 hours) allows making replicates in a short time, divers tester strains with several gene mutations allow to research the molecular effect mechanism of test materials, additional mutations in each strain result in more sensitivity such as *rfa* or *uvrB* and mesophile character of *Salmonella* allows to study several test materials affective at human body temperature. Furthermore, combination of the cytochrome-based P450 metabolic oxidation system, which usually consists of a 9000×g supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), with the *Salmonella* mutagenicity test allows to determine some mutagenic agents, which are biologically inactive unless they are metabolized to active forms (Ames et al., 1973b; Mortelmans & Zeiger, 2000; Zeiger et al., 2005). Thus, the assay is used world-wide in genetic toxicology laboratories as a beginning mutation test to determine mutagenic and antimutagenic potentials of various chemicals.

The present study includes an introduction to use of *Salmonella* strains in genetic toxicology, principles of *Salmonella* bacterial reverse mutation assay, the most popular assay procedures with explanatory figures and clues for experimental design.

## 2. Scientific background

### 2.1 Mutations and their effects on living organisms

Genetic materials of all living organisms are dynamic structures that change and rearrange many times as a result of cumulative effects of mutations. Mutations, described as sudden and spontaneous or induced changes in a genomic sequence, are classified in two main groups depend on their physical effect sizes on the genome (Hartl & Jones, 1998; Lewin, 2004; Klug et al., 2005; Brown, 2007). First group is large-scale mutations in chromosomal level, including numerical and structural anomalies. Numerical anomalies are also called as aneuploidy, means an abnormal number of chromosomes. The most known examples for aneuploidy are monosomy (missing a chromosome from a pair), nullisomy (missing a pair of chromosomes), trisomy and polysomy (having one or more than two chromosomes of a pair). Down and Turner syndromes are important examples related to chromosomal anomalies in humans. An individual with Down syndrome has a developmental disorder caused by having three copies of chromosome 21. Therefore, it is also called as Trisomy 21.

Another disorder is Turner syndrome, an example of a monosomy where the individual is born with only one X chromosome (Klug et al., 2005).

Structural anomalies of the large-scale mutations include deletions, duplications, inversions and translocations. A deletion is a loss of one or more pieces from a chromosome after DNA breaks induced by various physical or chemical agents (Klug et al., 2005). Genetic information loss together with deletions causes serious disorders in humans, for example, Wolf-Hirschhorn syndrome, also known as deletion 4p syndrome, and Jacobsen syndrome, also known as deletion 11q syndrome (Hirschhorn et al., 1965; Jacobsen et al., 1973). Duplication is described as a phenomenon that a chromosome has extra copies of a chromosomal region, which may affect phenotype by altering gene function and transcriptional dosage (Zhang, 2003; Mao & Pevsner, 2005). Because most embryonic processes requires sensitively balanced protein levels, many duplications lead to developmental defects such as *Bar* eye mutation in *Drosophila* and Charcot-Marie-Tooth disease in humans (Sutton, 1943; Latour et al., 1997). An inversion type chromosomal mutation occurs when a portion of the chromosome breaks off, 180° rotates and reattaches, resulting in an inverted genetic material. There is little knowledge about the linkage between inversions and disease formation, and it is believed that many affect mechanisms of inversions directly associated with deletions. Juvenile Polyposis of Infancy, a rare genetic disorder, is a good example for a disease evolved by the cumulative effects of inversions (a paracentric inversion in 10q) and deletions (a deletion in 10p) (Gimelli et al., 2003; Antonacci et al., 2009; Vargas-Gonzales et al., 2010). The last group of the structural anomalies is translocations, defined as an exchange of segments among the non-homologous chromosomes. Several forms of cancer, leukemia and lymphoma are the best known disorders related to translocations (Li et al., 1999; Kurzrock et al., 2003; Anton et al., 2004). Figure 1 illustrates structural chromosome mutations.

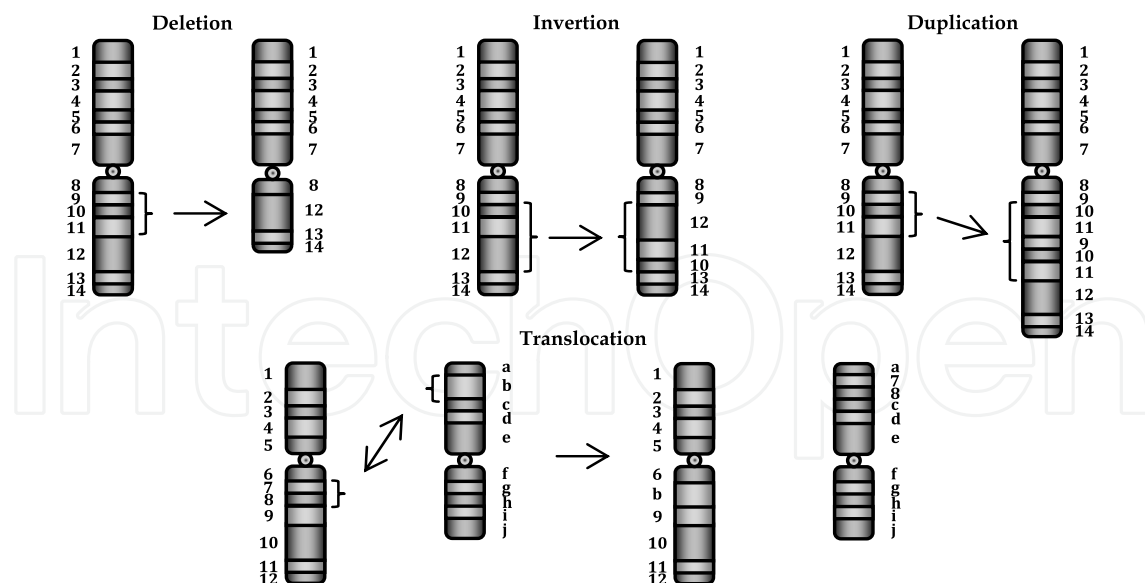


Fig. 1. Structural chromosome mutations

Small-scale mutations, also known as gene mutations, include three main groups: point mutations, which are the most common type of the gene mutations and replace one nucleotide with another, insertions, which add one or a few extra nucleotides into the DNA, and deletions, which remove one or a few nucleotides from the DNA (Brown, 2007).

Point mutations are also divided into two categories as transitions and transversions. Transitions, which are purine-to-purine or pyrimidine-to-pyrimidine changes ( $A \leftrightarrow G$  or  $C \leftrightarrow T$ ), are more common type of the point mutations than transversions, which are purine-to-pyrimidine or pyrimidine-to-purine changes ( $A \leftrightarrow C$ ,  $A \leftrightarrow T$ ,  $G \leftrightarrow C$  or  $G \leftrightarrow T$ ) (Brown, 2007).

Contrary to “small-scale” word in their names, these mutations can cause wide-range significant changes in genomes and phenotypes of living organisms with mutated genetic materials. For example, a point mutation may result in a synonymous change that causes forming a new codon specifying the same amino acid as the unmutated codon, a non-synonymous change that causes a missense mutation where a new codon specifies a different amino acid from the unmutated codon, a nonsense mutation where the change converts an amino acid specifying codon into a termination codon, or a readthrough mutation where the change converts a termination codon into an amino acid specifying codon. Except synonymous changes, also called as silent mutations because the mutated gene codes for exactly the same protein as the unmutated gene, the other three types of point mutations have significant impacts on the genome and related phenotypes by effecting amino acid sequence of the coding protein (Hartl & Jones, 1998; Alberts et al., 2002; Lewin, 2004; Brown, 2007; Lodish et al., 2007). The effects of point mutations on the coding region of a gene are shown in Figure 2.

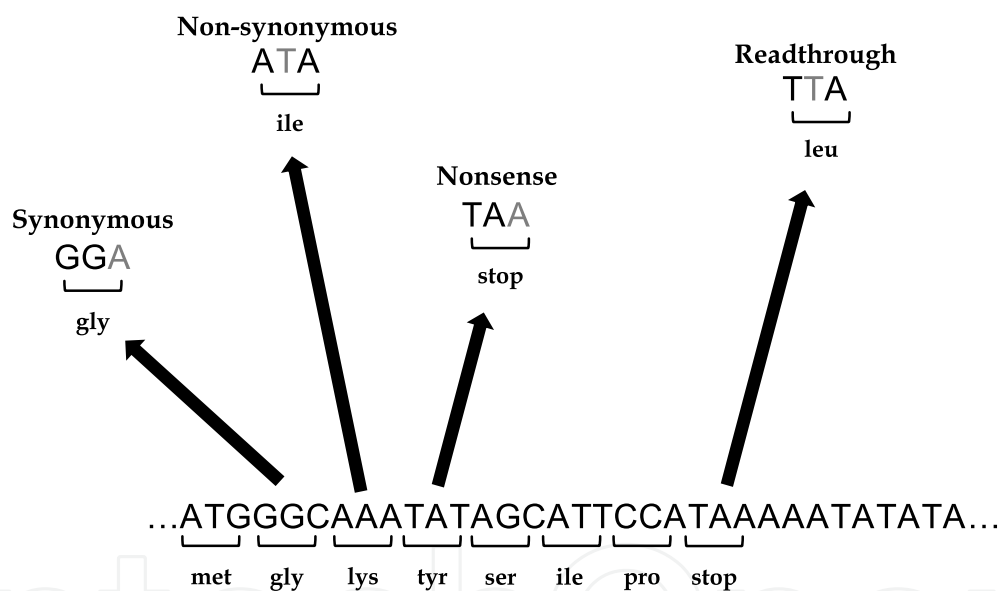


Fig. 2. The effects of point mutations on the coding region of a gene (Brown, 2007).

Insertion and deletion types of small-scale mutations affect the coding capabilities of the gene in a different way. It is defined as a frameshift mutation, caused by addition or deletion of a number of nucleotides that is not evenly divisible by three from a DNA sequence. Because codons consist of three nucleotides, an insertion or deletion type mutation can disrupt the reading frame, resulting in a completely different translation from the unmutated gene. Thus, insertion or deletion mutations generally have more significant effects on the protein function than the point mutations because the translated protein have completely different sequence from the mutated point to the end. An exception occurs that the number of inserted or deleted nucleotides is three or a multiple of three, which results in addition or deletion of one or more codons (Alberts et al., 2002; Lewin, 2004; Brown, 2007; Lodish et al., 2007). Figure 3 illustrates two possible effect mechanisms of the insertion or deletion type mutations on the coding region of a gene.

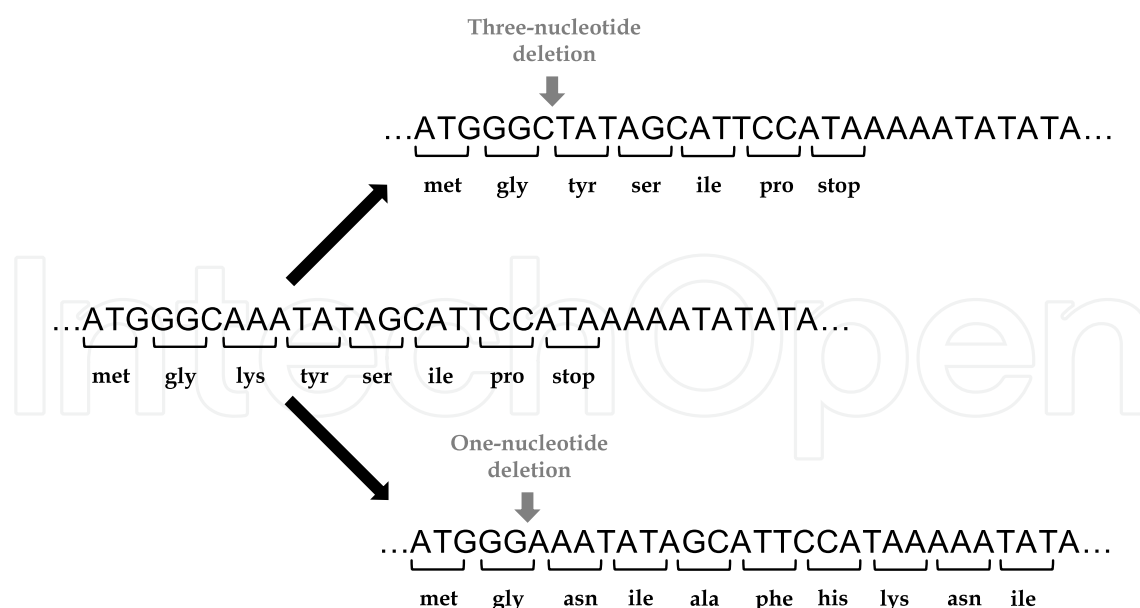


Fig. 3. Two possible effect mechanisms of the insertion or deletion type mutations on the coding region of a gene (Brown, 2007).

Phenotypic results of mutations can be deleterious or advantageous for the affected organism. Many hereditary disorders are either directly caused by the mutations or indirectly associated with the mutagenesis. Cancer formation can be given as a satisfactory example to demonstrate deleterious effect of mutations. Recent studies clearly showed that there is a strict connection between mutagenesis and the formation of the several cancer types (Davidson et al., 2002). In this manner, mutations provide a unique resource for all tumors that show genomic instability with few exceptions. On the other hand, mutated organisms can gain various advantages as a result of mutagenesis. Gain of antibiotic resistance in microorganisms and HIV/AIDS protective mutations on the SDF1, CCR5 and CCR2 genes in the human genome are well examples for the advantageous mutations (Stephan et al., 1998; Galvani & Slatkin, 2003; Apostolakis et al., 2005). These are also very important for evaluation of the organisms. Therefore, mutagenesis can be considered as one of the most important evolutionary sources. For example; simple sequence repeats (SSRs, also called microsatellites and minisatellites) are defined as advantageous mutators in adaptive evolution. Recent studies showed that temperature compensation of circadian rhythm in *Drosophila*, adaptive divergence among barley and wheat populations, social behavior in voles, skeletal morphology in domestic dogs and sporulation efficiency and cell adhesion in yeast are closely related to SSRs, which are mutation-prone DNA tracts composed of tandem repetitions of relatively short motifs (Kashi & King, 2006).

## 2.2 The causes of mutations

Mutations are divided into spontaneous and induced alterations according to their formation sources. Spontaneous mutations arise from replication errors due to defective replication enzymes and alternative tautomer forms of nucleotide bases. These are rare types of mutations. On the other hand, induced mutations, common types of mutations, are caused by various mutagens. In the molecular mechanism of induced mutations, a physical or chemical mutagen reacts with the DNA strand, causing a structural change that affects the base-pairing capability of the altered nucleotide. The most important types of physical



mutagens are ultraviolet radiation of wavelength 260 nm, ionizing radiation and heat shock. However, base analogs such as 5-bromouracil (5-bU), deaminating agents such as nitrous acid, alkylating agents such as ethylmethane sulfonate (EMS) and intercalating agents such as (ethidium bromide) are the most well-known chemical mutagen classes (Brown, 2007).

Chemical mutagens are more frequent agents because thousands of natural or synthetic chemicals have been introduced for daily use in many areas including medicine, pharmacy, food and cosmetics. The count of new chemicals is increasing day-by-day, and each chemical can be considered as a potential mutagen before tested. Therefore, many test systems for detecting of chemical mutagens have been developed and frequently used in the laboratories around the world (Zieger, 2000; World Health Organization [WHO], 2007).

### 2.3 The mutagenicity and antimutagenicity test systems

The deleterious effects of mutations enforce the determination of mutagenic chemicals. There are many assay systems for this purpose, and a new chemical is tested for mutagenic potential before introduced to use. The main groups of the assay systems are long-term and short-term assay systems (Wickramasnghe, 1979; Zeiger et al., 2005; Mortelmans & Zeiger, 2000).

The long-term assay systems mainly include *in vivo* applications performed with experimental animals. These are the most comprehensive and reliable test systems. However, the long-term assay systems are not preferred as the beginning mutation test systems due to their high – cost and time consuming properties (Wickramasnghe, 1979; Zeiger et al., 2005).

On the other hand, the short-term assay systems mainly include *in vitro* assays performed with bacterial strains, cytological cell-line cultures and biotechnology based applications. Relatively inexpensive and time-saver nature of the short-term assay systems makes them good candidates for preliminary mutagen determination studies performed with fairly huge numbers of synthetic or natural chemicals. Furthermore, these assays can identify substances inhibiting mutagens and mutations (called as antimutagens) with some modifications. Therefore, a mutagenicity test system can be also considered as antimutagenicity test system (Ames et al., 1973a, 1973b; Wickramasnghe, 1979; Fenech, 2000; Maron & Ames, 1983; Mortelmans & Riccio, 2000; Mortelmans & Zeiger, 2000; Zeiger et al., 2005; Rossi et al., 2007; Ozbek et al., 2008a, 2008b; Gulluce et al., 2010).

### 2.4 Ames/Salmonella test system

The Ames/*Salmonella* test system, also called as Ames test, was developed by Ames *et al.* in the beginning of 1970s. The test, which employs histidine auxotroph *Salmonella* strains originated from *Salmonella typhimurium* LT-2 by chemical and radiation induced mutations, was initially designed as a spot test for determination of mutagenic chemicals, then as a more sensitive method: plate incorporation test (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Gee et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

In the molecular mechanism of the test system, a tester strain carries a unique gene mutation at the histidine operon that makes the strain histidine-dependent to grow, and a mutagenic chemical reacts with the mutated site resulting in a reverse mutation. Thus, the strain regains histidine production ability and the bacterial cells can grow in the absence of histidine. Therefore, the test is often referred as a reversion assay (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Gee et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

After its introduction to the scientific world, Ames test has been widely accepted as a short-term bacterial test system for determining chemicals that can cause gene mutations. The test has many advantages for identification of the chemicals that cause gene mutations. These advantages can be listed in:

- Short-term resulting: allows making replicates and obtaining more reliable results in a short duration. It takes only about 48 hours.
- Low-cost: allows studying a large number of test materials inexpensively.
- Various tester strains with several gene mutations: enable to research the molecular effect mechanism of test materials
- Additional mutations and genetic alterations: allow gaining more sensitivity for various chemicals.
- Mesophile character of *Salmonella* strains: enables to study mutagenic potential of the chemicals at human body temperature.

Apart from all the maintained advantages, Ames/*Salmonella* test system is very versatile, and many modifications has been developed to determine mutagenic potencies of various materials such as environmental chemicals, environmental mixtures, body fluids, foods, drugs and physical agents. The most common assay procedures are the spot test: a primal method for determination of chemical mutagens, the standard plate incorporation method: an easily resulting and more comprehensive method than the spot test, the pre-incubation method: developed for performing more effective studies with lower volumes of test materials, the desiccator assay modifications: developed to study volatile materials and gases, and the modified *Salmonella* microsuspension assay (Kado): a highly sensitive method for testing the materials that are available only in small amounts (Kado et al., 1983; Hughes et al., 1987; Zeiger et al., 1992; Araki et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

Although *Salmonella* has prokaryotic cell structure, combination of the cytochrome-based P450 metabolic oxidation system with the Ames/*Salmonella* test system allows determining some mutagenic agents, which are biologically inactive unless they are metabolized to active forms (Ames et al., 1973b; Mortelmans & Zeiger, 2000). Moreover, all the procedures of the test system can be altered to identify antimutagenic agents, inhibit mutagenesis and protect the organisms against deleterious effects of the mutagens, with some modifications (Nagabhushan et al., 1987; Bala & Grover, 1989; Edenharder et al., 1999; Edenharder & Grünhage, 2003; Ozbek et al., 2008a, 2008b; Gulluce et al., 2010) (see 3.8).

### 3. Material and methods

#### 3.1 Supplies and equipment

The following items are required for performing the Ames/*Salmonella* mutagenicity and antimutagenicity protocols.

##### 3.1.1 Supplies

- Magnetic stir bars
- Sterile glass test tubes (100×16 mm) and racks
- Sterile microbiological loops
- Sterilizing membrane filters (0.2 μm)



- Sterile syringes (5, 10 and 50 ml)
- Sterile Petri dishes (100×15 mm)
- Disposable spectrophotometer cuvettes
- Solvents, reagents, media and positive control chemicals
- General laboratory glassware (bottles, flasks and graduated cylinders)
- Dispensers for delivering top agar, buffer and S-9 mix to the test tubes
- Sterile cryogenic storage vials for freezing down permanent and working cultures
- General laboratory safety items (biohazard waste bags, goggles or protective eye wear, gloves, lab coats)
- Glass pipettes (1, 2, 5 and 10 ml), automatic micropipettes (adjustable volumes up to 200 and 500  $\mu$ l) and pipette tips

### 3.1.2 Equipment

- Autoclave
- Manual or electronic colony counter
- Spectrophotometer for monitoring cell density
- Centrifuge (up to 8000 rpm)
- Liquid and solid waste disposal
- Magnetic stirrers
- Desiccator and vacuum pump
- Balances
- Biological/chemical safety cabinet equipped with gas line for keeping aseptic techniques while inoculating cultures
- Ultra-low temperature freezer set at  $-86\text{ }^{\circ}\text{C}$  or liquid nitrogen tank for long term storage of frozen permanent cultures
- Refrigerator ( $4\text{ }^{\circ}\text{C}$ ) and freezer ( $-20\text{ }^{\circ}\text{C}$ )
- Water purification system to generate distilled water
- Water bath set at  $43\text{ }^{\circ}\text{C}$  to  $48\text{ }^{\circ}\text{C}$  to maintain temperature of top agar
- Incubator for incubating the agar plates
- Shaking incubator for incubating the liquid cultures and growing the overnight cultures
- Boiling water bath or microwave oven for melting top agar

### 3.2 Reagents and media

*Glucose solution (10% w/v)*: The solution is used as carbon source for the GM agar plates. Dissolve 100 g dextrose (D-glucose) in 700 ml of distilled water by stirring on a magnetic stirrer. Add additional water to bring the final volume to 1000 ml and distribute in 50 ml aliquots. Autoclave  $121\text{ }^{\circ}\text{C}$  for 20 min and store at  $4\text{ }^{\circ}\text{C}$ .

*Vogel-Bonner medium E (VB salts 50 $\times$ )*: The solution is used as salt source for the GM agar plates. Add 10 g magnesium sulfate ( $\text{MgSO}_4\cdot\text{H}_2\text{O}$ ), 100 g citric acid monohydrate ( $\text{C}_6\text{H}_8\text{O}_7\cdot\text{H}_2\text{O}$ ), 500 g potassium phosphate dibasic ( $\text{K}_2\text{HPO}_4$ ) and 175 g sodium ammonium phosphate ( $\text{Na}_2\text{NH}_2\text{PO}_4\cdot 4\text{H}_2\text{O}$ ) in the order indicated to 650 ml of warm water making sure that each salt is dissolved thoroughly by stirring before adding the next salt. Add additional water to bring the final volume to 1000 ml and distribute in 20 ml aliquots. Autoclave  $121\text{ }^{\circ}\text{C}$  for 30 min and store at room temperature in the dark.

*GM agar plates:* The medium is used as bottom agar for mutagenicity and antimutagenicity assays. Add 15 g agar to 900 ml of distilled water and autoclave for 30 min at 121 °C. When cooled to approximately 65 °C, add 20 ml of sterile Vogel-Bonner medium E and mix well; then add 50 ml of sterile glucose solution and mix thoroughly. Pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes and store at 4 °C for several weeks by packing with sealed plastic bags after solidified. Note that the plates should be warmed up to room temperature and examined for excess moisture before use. Put the plates with too much moisture overnight in an incubator set at 37 °C prior to use.

*Histidine/biotin solution (0.5 mM):* The solution is used to supplement top agar with adequate biotin and a trace amount of histidine. Dissolve 124 mg D-biotin and 96 mg L-histidine·HCl in 1000 ml of boiling water. Sterilize the solution by filtration through a membrane filter with 0.2 µm pore size or autoclaving for 20 min at 121 °C. Store at 4 °C in a glass bottle.

*Top agar supplemented with histidine/biotin:* The solution is used to apply the bacteria, chemicals and buffer or S9 mix to the bottom agar. Dissolve 6 g agar and 6 g sodium chloride (NaCl) in 900 ml of distilled water by heating. Add 100 ml of histidine/biotin solution (0.5 mM) and dispense 200 ml aliquots in screw-cap bottles. Autoclave for 20 min at 121 °C and store at room temperature in the dark. Melt the top agar in a microwave oven or boiling water bath before use.

*Nutrient broth:* Oxoid nutrient broth no. 2 or Difco nutrient broth can be used to grow the tester strains overnight. Follow the manufacturer's instructions for preparing the medium. Dispense 50 ml in Erlen Meyer flasks with 125 ml capacity or 5 ml in 100×16 mm test tubes, autoclave for 20 min at 121 °C and store in the dark at room temperature.

*Nutrient agar plates:* The medium is used for streaking newly received cultures for single colonies, checking crystal violet sensitivity due to presence of *rfa* mutation and testing viability of bacteria. Add 15 g agar to 1000 ml of nutrient broth medium and dissolve by heating. Autoclave for 20 min at 121 °C. After cooled to 65 °C, pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes and store at 4 °C by packing with sealed plastic bags.

*Sodium phosphate buffer (0.1 mM – pH 7.4):* The solution is used to perform mutagenicity and antimutagenicity assays in the absence of metabolic activation. In the first step, prepare Reagent A (0.1 M sodium phosphate monobasic solution: 13.8 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O in 1000 ml of distilled water) and Reagent B (0.1 M sodium phosphate dibasic reagent: 14.2 g Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O in 1000 ml of distilled water). After that, mix 120 ml of Reagent A and 880 ml Reagent B and swirl well. Adjust pH to 7.4 using Reagent A/B and dispense 100 ml aliquots in screw-cap bottles. Autoclave for 30 min at 121 °C and store at room temperature in the dark.

*Metabolic activation system (S-9 mix):* The solution is used to perform mutagenicity and antimutagenicity assays in the presence of metabolic activation. Moltox metabolic activation system products can be used. Follow the manufacturer's instructions for preparation and storage of the related solutions.

*Biotin solution (0.01%, w/v):* The solution is used to prepare enriched GM agar plates for biotin auxotrophy check. Dissolve 10 mg D-biotin in 100 ml of boiling distilled water. Sterilize using a membrane filter with 0.2 µm pore size and store at 4 °C.

*Histidine solution (0.5%, w/v):* The solution is used to prepare enriched GM agar plates for histidine auxotrophy check. Dissolve 500 mg L-histidine in 100 ml of distilled water. Autoclave for 15 min at 121 °C and store at 4 °C.

*Ampicillin solution (0.8%, w/v)*: The solution is used to prepare enriched GM agar plates for examining presence of plasmid pKM101 in several tester strains such as TA97, TA98, TA100 and TA102. Dissolve 8 mg ampicillin in 100 ml of warm (65 °C) distilled water and sterilize using membrane filter with 0.2 µm pore size. Store at 4 °C.

*Tetracycline solution (0.8%, w/v)*: The solution is used to prepare enriched GM agar plates for examining presence of plasmid pAQ1 in TA102. Dissolve 8 mg tetracycline in 100 ml of 0.02 N hydrochloric acid (HCl) and sterilize using membrane filter with 0.2 µm pore size. Store at 4 °C in the dark due to the light sensitivity of tetracycline.

*Enriched GM agar plates*: Each medium contains essential nutrients and antibiotics for the strain check and preparation of stock cultures' master plates.

- Biotin plates (B): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Histidine plates (H): Prepare GM agar medium. After autoclaving, add 8 ml of sterile histidine solution (0.5%, w/v), mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine plates (BH): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v) and 8 ml of sterile histidine solution (0.5%, w/v), mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine/ampicillin plates (BHA): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), 8 ml of sterile histidine solution (0.5%, w/v) and 3 ml of ampicillin solution, mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine/tetracycline plates (BHT): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), 8 ml of sterile histidine solution (0.5%, w/v) and 0.25 ml of tetracycline solution, mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine/ampicillin/tetracycline plates (BHAT): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), 8 ml of sterile histidine solution (0.5%, w/v), 3 ml of ampicillin solution and 0.25 ml of tetracycline solution, mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.

*Crystal violet solution (0.1%, w/v)*: The solution is used to confirm the presence of the *rfa* mutation in all of the tester strains. Dissolve 100 mg crystal violet in 100 ml of distilled water. Mix well and store at 4°C in an amber glass bottle to protect against light.

All reagents and solutions, reported here, have been previously described by Mortelmans and Zeiger (2000).

### 3.3 Bacterial strains

*Salmonella typhimurium* TA1535, TA1537, TA1538, TA97, TA98, TA100, TA102 and TA104 are the most common tester strains used in the Ames/*Salmonella* test system. All strains are histidine auxotroph because of a mutation in the histidine operon. The tester strains also have additional mutations and genetic alterations that provide more sensitivity for chemical mutagens. These are *uvrB*, *rfa* mutations and introduction of pKM101 and pAQ1 plasmids.

The *uvrB* mutation, which is present in all strains except TA102, arise from a deletion type mutation through the *uvrB-bio* genes that eliminates the accurate DNA repair and makes the cells biotin dependent. All strains have the *rfa* mutation that affects the bacterial cell wall, resulting in a defective lipopolysaccharide layer that provides more permeability to bulky chemicals. Existence of pKM101 plasmid in TA97, TA98, TA100, and TA102 provides ampicillin resistance and sensitivity for chemical and induced mutagenesis associated with error-prone recombinational DNA repair pathway. TA102 strain also has multicopies of pAQ1 plasmid carrying *hisG428* mutation, which provides tetracycline resistance and sensitivity for detection of DNA cross-linking agents (Mortelmans & Zeiger, 2000). Table 1 presents genotypes of the tester strains.

Strain	DNA Target	<i>uvrB</i>	<i>rfa</i>	Plasmid	Reversion Event
TA1535	<i>hisG46</i> -G-G-G-	+	+	-	Base-pair substitutions
TA1537	<i>hisC3067</i> -C-C-C- +1 frameshift	+	+	-	Frameshifts
TA1538	<i>hisD3052</i> -C-G-C-G-C-G-C-G- -1 frameshift	+	+	-	Frameshifts
TA97	<i>hisD6610</i> -C-C-C-C-C-C- +1 frameshift	+	+	pKM101	Frameshifts
TA98	<i>hisD3052</i> -C-G-C-G-C-G-C-G- -1 frameshift	+	+	pKM101	Frameshifts
TA100	<i>hisG46</i> -G-G-G-	+	+	pKM101	Base-pair substitutions
TA102	<i>hisG428</i> TAA (ochre)	-	+	pKM101 pAQ1	Base-pair substitutions
TA104	<i>hisG428</i> TAA (ochre)	+	+	-	Base-pair substitutions

Table 1. Genotypic properties of the most common *Salmonella* tester strains.

There are also additional tester strains (TA7001-7006 and TA7041-7046 series), which developed by Gee et al. (1994), to identify specific transitional and transversional base-pair substitutions induced by various mutagenic agents. Table 2 presents genotypic properties of these strains.

Strain	DNA Target	<i>uvrB</i>	<i>rfa</i>	Plasmid	Reversion Event	Amino acid change
TA7001	<i>hisG1775</i>	+	+	pKM101	AT → GC	Asp-153 → Gly-153 (GAT → GGT)
TA7002	<i>hisC9138</i>	+	+	pKM101	TA → AT	Ile-217 → Lys-217 (ATA → AAA)
TA7003	<i>hisG9074</i>	+	+	pKM101	TA → GC	Val-153 → Gly-153 (GTT → GGT)
TA7004	<i>hisG9133</i>	+	+	pKM101	GC → AT	Gly-169 → Asp-169 (GGG → GAT)
TA7005	<i>hisG9130</i>	+	+	pKM101	CG → AT	Ala-169 → Asp-169 (GCG → GAT)
TA7006	<i>hisC9070</i>	+	+	pKM101	CG → GC	Arg-163 → Gly 163 (CGA → GGA)
TA7041	<i>hisG1775</i>	+	-	pKM101	AT → GC	Asp-153 → Gly-153 (GAT → GGT)
TA7042	<i>hisC9138</i>	+	-	pKM101	TA → AT	Ile-217 → Lys-217 (ATA → AAA)
TA7043	<i>hisG9074</i>	+	-	pKM101	TA → GC	Val-153 → Gly-153 (GTT → GGT)
TA7044	<i>hisG9133</i>	+	-	pKM101	GC → AT	Gly-169 → Asp-169 (GGG → GAT)
TA7045	<i>hisG9130</i>	+	-	pKM101	CG → AT	Ala-169 → Asp-169 (GCG → GAT)
TA7046	<i>hisC9070</i>	+	-	pKM101	CG → GC	Arg-163 → Gly 163 (CGA → GGA)

Table 2. Genotypic properties of *Salmonella* tester strains developed by Gee et al. (1994).

The test system performed with TA700x tester strains is called as AMES II (Kamber et al., 2009). The set of TA7041-7046 strains is not suitable to test mutagenic and antimutagenic potential of chemicals due to lack of *rfa* mutation and their instable genotypes.

### 3.4 Positive control chemicals

Chemicals divide in two groups according to their affect mechanisms. These groups are direct and indirect acting positive controls.

Many direct acting agents has been introduced as positive controls because of their high specificity for the tester strains. The most common direct-acting positive control chemicals for Ames/*Salmonella* test system are listed in Table 3.

2-Aminoanthracene (2-AA; CAS# 613-13-8), 2-Aminofluorene (2-AF; CAS# 153-78-6) and Aflatoxin B1 (AFB1; CAS# 1162-65-8) are frequently used indirect-acting positive controls that requires metabolic activation before react with the *Salmonella* tester strains (Mortelmans & Zeiger, 2000; Ozbek et al., 2008b; Limem et al., 2010).



Chemical	Mechanism of Genotoxicity	Tester Strain	Reference	CAS#
4-Nitro- <i>o</i> -phenylenediamine (4-NPD)	Teratogenic and intercalating agent	TA1538 TA98	Ben Sghaier et al., (2010) Kaur et al., (2010) Mortelmans & Zeiger (2000)	99-56-9
4-Nitroquinoline 1-oxide (4-NQO)	Causing DNA lesions	TA1538 TA100 TA98	Oh et al., (2008) Ozbek et al., (2008a) Brennan & Schiestl (1998)	56-57-5
9-Aminoacridine (9-AA)	DNA intercalating agent	TA1537 TA97	Gulluce et al., (2010) Miadokova et al., (2009) Mortelmans & Zeiger (2000)	90-45-9
Methyl methane sulfonate (MMS)	Alkylating agent	TA102 TA104	Mortelmans & Zeiger (2000) Dellai et al., (2009) Zahin et al., (2010)	66-27-3
Mitomycin C (MTC)	DNA cross-linker and alkylating agent	TA102	Biso et al., (2010) Mortelmans & Zeiger (2000) Zhang et al., (2011)	50-07-7
<i>N</i> -Methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine (MNNG)	Alkylating agent	TA100	Caldini et al., (2005) Duh et al., (2009) Oh et al., (2008)	70-25-7
Sodium azide (NaN <sub>3</sub> )	L-azidoalanine mediated base substitution	TA1535 TA100	Bulmer et al., (2007) Gulluce et al., (2010) Mortelmans & Zeiger (2000)	26628-22-8

Table 3. The frequently used direct-acting chemicals for the *Salmonella* tester strains.

### 3.5 Genetic analysis of the *Salmonella* tester strains

When a new strain received, its genotypic characteristics (*his*, *rfa* and *uvrB-bio*), spontaneous mutation rate and the presence of pKM101 and pAQ1 plasmids should be checked before preparation of frozen cultures for long term storage. For this purpose, follow these steps:

#### 3.5.1 Inchoative stages for genetic analysis

- Add 1 mL of sterile nutrient broth to rehydrate the lyophilized culture.
- Transfer 10  $\mu$ L of the rehydrated culture to nutrient agar plate and stroke the inoculum to get individual colonies that serve as main sources for the genetic analysis of the tester strains.
- Transfer the rest portion of the rehydrated culture to 4 mL of nutrient broth. This broth culture serve as a back-up point in case of there is no growth on the nutrient agar plates.
- Incubate the cultures overnight at 37 °C. Then, check the agar plates and broth cultures for bacterial growth.

- At least two purification steps should be made to get more reliable results. Pick one healthy looking colony and streak it again on nutrient agar plates or GM agar plates supplemented with excess of biotin and histidine. If the tester strain carries pKM101 and/or pAQ1 plasmids, GM agar plates should be also supplemented with ampicillin and/or tetracycline, respectively. However, growth of the tester strains on the supplemented GM agar plates takes more time (approximately 48 h) than nutrient agar plates; it is recommended because using of them reduces contamination risks.

### 3.5.2 Genetic analysis

Five mandatory steps for all strains and additional one or two steps for plasmid carrying strains should be made to perform the best reliable genetic analysis. For this purpose, inoculate 5 mL of nutrient broth with a single colony after purification steps, and incubate the culture overnight 37 °C. Then, follow these steps for a complete strain check:

- In the 1<sup>st</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin, which demonstrates the histidine dependence (*his* mutation) of all the *Salmonella* tester strains. After an incubation period at 37 °C for 24-48 h, there should be no growth on the plate (see Figure 4a).
- In the 2<sup>nd</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of histidine, which demonstrates the biotin dependence (*bio* mutation) of all the *Salmonella* tester strains except TA102 strain. After an incubation period at 37 °C for 24-48 h, there should be no growth on the plate. Due to lack of the *bio* mutation, TA102 strain can be growth on a GM agar plate supplemented with excess of histidine (see Figure 4b).
- In the 3<sup>rd</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin and histidine, which demonstrates the biotin and histidine dependence (*bio* and *his* mutations) of all the *Salmonella* tester strains. After an incubation period at 37 °C for 24-48 h, there should be growth on the plate (see Figure 4c).
- In the 4<sup>th</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin and histidine. Place a sterile filter paper disk in the middle of the plate and apply 10 µL crystal violet solution (0.1%, w/v) onto the disk. After an incubation period at 37 °C for 24-48 h, all strains show a zone of growth inhibition surrounding the disk, which demonstrates the presence of *rfa* mutation (see Figure 4d).
- In the 5<sup>th</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin and histidine. Unseal the top and cover the half of the plate with sterile aluminum foil. Expose the plate to a low level of UV irradiation for a short time (approx. 8-10 seconds) that kills the *uvrB* strain but not its isogenic DNA repair proficient strain. After an incubation period at 37 °C for 24-48 h, there should be normal growth on the non-exposed part of the plate but not on the exposed part. It demonstrates the presence of *uvrB* mutation. It is known that the source of *uvrB* mutation, a deletion mutation, also covers the biotin gene region. Therefore, if a strain shows a positive *bio* mutation result in the 2<sup>nd</sup> step, there is no need to check the presence of the *uvrB* mutation for this strain (see Figure 4e).
- In the 6<sup>th</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin, histidine and ampicillin. After an incubation period at 37 °C for 24-48 h, there should be growth on the plate, which demonstrates the

presence of pKM101 plasmid in the tester strains TA97, TA98, TA100, TA102 and TA104 (see Figure 4f).

- In the 7<sup>th</sup> step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of histidine and tetracycline. After an incubation period at 37°C for 24-48 h, there should be growth on the plate, which demonstrates the presence of pAQ1 plasmid in the tester strain TA102 (see Figure 4f).

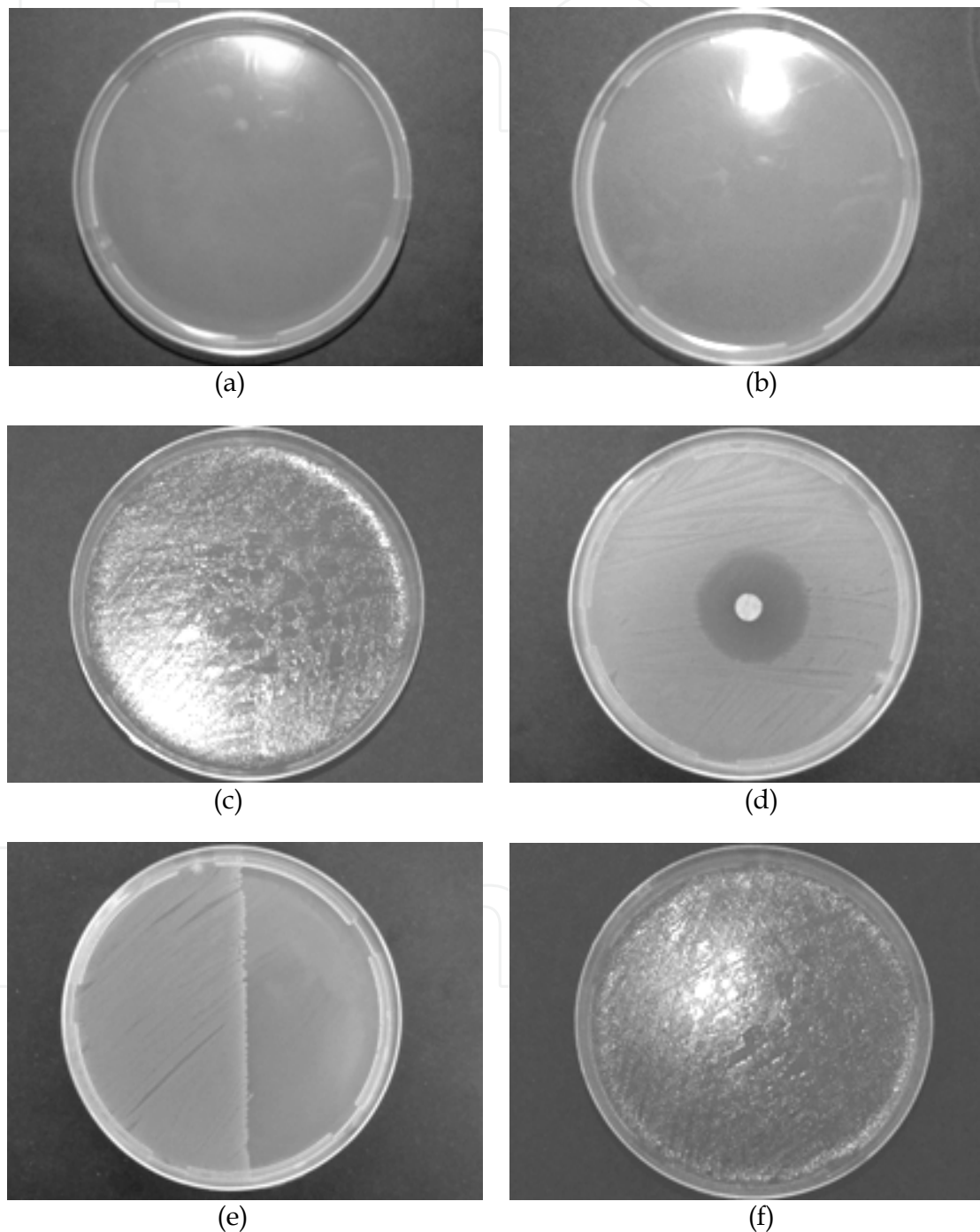


Fig. 4. Demonstration of (a) histidine, (b) biotin and (c) biotin/histidine dependence of the *Salmonella* tester strains, and presence of (d) the *rfa* mutation, (e) the *uvrB* mutation, (f) pKM101/pAQ1 plasmids.

### 3.5.3 Spontaneous mutation rates

Each laboratory has its characteristic spontaneous mutation rates for the all tester strains, and these values show a wide-range variation among the laboratories. Therefore, the spontaneous mutant frequency should be determined for all strains and recorded. It serves as historical control values provide choosing suitable strains for mutagenicity and antimutagenicity assays. Table 4 shows a sample of acceptable control values for the most common *Salmonella* tester strains.

Strain	Number of revertants	
	Without metabolic activation	With metabolic activation
TA97	75-200	100-200
TA98	20-50	20-50
TA100	75-200	75-200
TA102	100-300	200-400
TA104	200-300	300-400
TA1535	5-20	5-20
TA1537	5-20	5-20
TA1538	5-20	5-20

Table 4. Spontaneous revertant control values for the most common *Salmonella* tester strains (Mortelmans & Zeiger, 2000).

### 3.6 Long term storage of the tester strains

The *Salmonella* tester strains should be stored in a freezer at -80 °C or liquid nitrogen. Healthy looking single colonies should be chosen to prepare the frozen stock cultures. Dimethylsulfoxide (DMSO) or glycerol is suggested as cryoprotective agent. The final concentration of the cryoprotective should be at least 10% (v/v) (Mortelmans & Zeiger, 2000).

### 3.7 Viability assay and determination of test concentrations

Cytotoxic properties of the test materials toward the *Salmonella* tester strains should be determined before performing mutagenicity and antimutagenicity assays. The viability assay includes observations for *Salmonella* colonies on plates after 48 h incubation at 37 °C. Following three main characteristics for the tester strains should be taken into account.

- Thinning of the background lawn
- Absence of background lawn
- Presence of pinpoint non-revertant colonies

These characteristics indicate toxic levels of the test chemicals, and applicable dose ranges should be determined by repeating of the viability assay with lower concentrations of the test chemicals (Mortelmans & Zeiger, 2000).

### 3.8 Mutagenicity and antimutagenicity assays

Various test procedures for Ames/*Salmonella* test system have been developed to determine mutagenic and antimutagenic potency of synthetic and natural chemicals. These procedures mainly are based on the physical properties or quantity of the test chemical. For example; the desiccator assay has been developed for gases and volatile substances, and Kado assay allows studying the chemicals in small amounts. However, the standard plate incorporation method is the most common application procedure of the Ames/*Salmonella* test system (Kado et al., 1983; Hughes et al., 1987; Zeiger et al., 1992; Araki et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

#### 3.8.1 The standard plate incorporation method

The method consists of exposing the tester strains to the test chemical directly on a glucose agar plate. The main advantages of the method can be listed in giving easy, reproducible, reliable and comprehensive results.

Follow these steps for performing mutagenicity assay (Mortelmans & Zeiger, 2000):

1. Steps taken prior to performing the experiment
  - Inoculate the tester strain from frozen culture into 5 mL of nutrient broth and incubate the new culture overnight at 37 °C.
  - Prepare an appropriate number of labeled GM agar plates and sterile tubes for each test chemical.
  - Prepare metabolic activation system and keep on ice until use.
  - Prepare chemical dilutions.
  - Melt top agar supplemented with 0.05 mM histidine and biotin and maintain at 43 °C to 48 °C.
2. Add following items respectively into sterile glass tubes maintained at 43 °C and mix well each addition\*.
  - 2 mL of molten top agar
  - 0.5 mL of S-9 mix (for the test performed with metabolic activation system) or buffer (without activation)
  - 0.05 mL of the test chemical dilution
  - 0.05-0.10 mL overnight culture of the tester strain (approx.  $1-2 \times 10^8$  bacteria per tube -  $A_{540}$  0.1-0.2)
3. Mix well the tubes and pour onto the surface of GM agar plates
4. When the top agar is solidify, invert and incubate the cultures at 37 °C for 48 h
5. Count the colonies after incubation and express the results as the number of revertant colonies per plate.

\*Notes: This step includes two additional groups which are negative controls and positive controls. The negative controls do not include 0.05 mL of the test chemicals, but include the solvent at equal quantity. Positive controls also do not include 0.05 mL of the test chemicals, but include the suitable positive mutagen solution for the tester strain at equal quantity.

The procedures of mutagenicity assay are all applicable to the antimutagenicity assay. The only procedural difference is the addition of the suitable positive mutagen solution to the all test chemical groups (Nagabhushan et al., 1987; Bala & Grover, 1989; Edenharder et al., 1999; Edenharder & Grünhage, 2003; Ozbek et al., 2008a, 2008b; Gulluce et al., 2010).



#### 4. Conclusion

In conclusion, the mutant *Salmonella* strains are beneficial for humanity contrary to their pathogenic wild-type strains. The histidine auxotrophic *Salmonella typhimurium* strains, object of the present study, provide a possibility to determine natural and synthetic chemicals with mutagenic properties. Similarly, these are also valuable for identification of antimutagenic chemicals after minor technical modifications. When a chemical, precious for industrial or health applications, is found or synthesized, determination of its genotoxic properties has a great importance. In this perspective, the Ames test allows making relatively cheap and reliable applications resulting in a short time.

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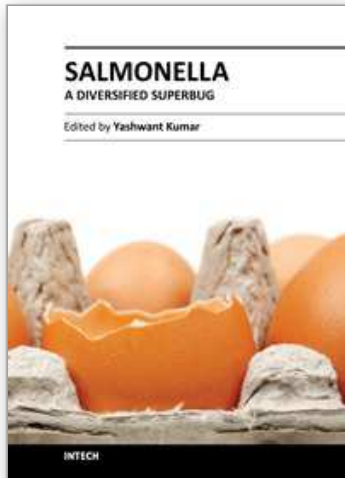
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## **Salmonella - A Diversified Superbug**

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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