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UMI

A STUDY OF THE ARABINOSE FORWARD MUTATION SYSTEM OF

SALMONELLA TYPHYMURIUM UNDER AN ANAEROBIC ENVIRONMENT

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

SUSHAMA VARMA

August 1995

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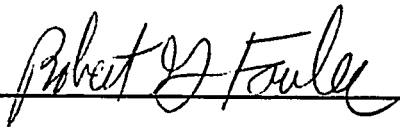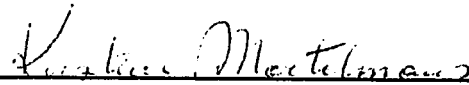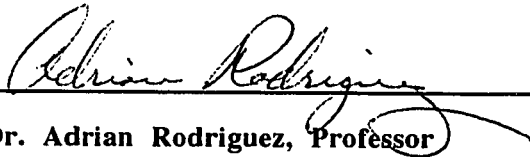
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Abstract

A STUDY OF THE ARABINOSE FORWARD MUTATION SYSTEM OF

SALMONELLA TYPHIMURIUM UNDER AN ANAEROBIC ENVIRONMENT

by

SUSHAMA VARMA

The aim of the present work is to evaluate the *Salmonella typhimurium*

Ara^R forward mutation system as an indicator of mutagenicity in an anaerobic environment.

Mutagenesis was measured by selecting for forward mutations to L-arabinose resistance

under both aerobic and anaerobic conditions. Both spontaneous and EMS-induced mutation

frequencies were measured. EMS increases mutation frequencies in both environments;

however an average 5-fold increase in the EMS-induced mutation frequency was seen

under aerobic conditions when compared to an anaerobic environment. The data show an

average 9-fold decrease in the spontaneous mutation frequency to Ara^R in the anaerobic

environment when compared to aerobic values. These data suggest that oxygen is

responsible for a major component of both spontaneous and EMS-induced mutagenesis and

the Ara^R forward mutation system can be used to measure mutation frequencies

anaerobically.

To: Shobha Varma, Puspha Dhar and Meghana Dhar for my inspiration and their unconditional love and support.

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Introduction

Bacterial mutation assays are sensitive, rapid and inexpensive. These assays can efficiently screen chemicals which are potential mutagens and carcinogens. The most widely used system is the *Salmonella typhimurium* histidine reversion assay. A set of *S. typhimurium* strains that carry specific lesions in the histidine operon which must be reverted or suppressed in order to produce histidine prototrophy is used in this assay.

An alternative approach to a reversion assay is the forward mutation L-arabinose assay of *S. typhimurium*. In this system a strain is used that carries the *araD531* mutation which blocks the metabolism of L-arabinose leading to the accumulation of a toxic intermediate, thus inhibiting bacterial growth. The assay selects changes from L-arabinose sensitivity (Ara^S) to L-arabinose resistance (Ara^R).

The purpose of this study was to examine the response of the L-arabinose-resistance system to spontaneous and induced mutation frequencies under aerobic and anaerobic conditions. An anaerobic chamber with a built-in incubator which allows the maintenance of anaerobic conditions at all stages of culturing anaerobic microorganisms was used. A known chemical mutagen, ethyl methanesulfonate (EMS), was used to measure induced mutation frequencies under aerobic and anaerobic conditions as it is believed that oxygen affects mutagenesis.

Literature Review

Environmental mutagens and carcinogens may have two major effects in humans: They can cause mutations in the germ cells resulting in the accumulation of heritable changes in the gene, and they may cause mutations in somatic cells giving rise to the formation of malignancies in individuals. It is known that a single point mutation, a GC to TA transversion, results in the activation of a human proto-oncogene (Tabin et al., 1982).

A large number of short-term tests have been developed to test environmental chemicals for their mutagenicity and carcinogenicity (Venitt and Parry, 1984). Some of the major short-term tests use prokaryotes such as bacteria and phage. Eukaryotic short-term tests have been developed in yeast (Zimmerman, 1975), *Drosophila* (Wurgler et al., 1977) and mammalian cell lines. Mammalian short-term tests include assays for the induction of DNA repair (reviewed in Maher et al., 1976; Lieberman, 1978), assays for the detection of chemically-induced chromosomal damage (Hollstein and McCann, 1979) and assays for detection of point mutations (Hollstein and McCann, 1979)). Batteries of tests can be used because of the rapid growth of cells, the large number of cells that can be treated and the fact that these assays are simple and inexpensive (Hollstein and McCann, 1979).

The short-term tests using bacteria and phage can be divided into four categories depending upon the detecting system used to test the chemicals:
(1) Reversion in auxotrophs - These test systems detect changes from auxotrophy to prototrophy. The changes involve specific point mutations in the DNA, such as base-pair

substitutions or frameshift mutations. These systems utilize different strains of auxotrophic bacteria. For example, *Escherichia coli* B strain WP-2 is a tryptophan auxotroph which detects base-pair substitution mutagens which increase the frequency of Trp^+ prototrophs. It is especially useful for detecting weak alkylating agents (Green and Muriel, 1976). *E. coli* K-12 strain 343/113 (lambda) requires galactose, nicotinic acid and arginine for growth due to three mutant genes. This assay detects reversion to prototrophy at the above three loci by base-pair substitution or frameshift mutagens. It can also detect forward mutation to 6-methyl tryptophan resistance or loss of galactose-fermenting capability and induction of prophage (Mohn and Ellenberger, 1977). The *Salmonella*/Ames test uses several *Salmonella* strains which require histidine for growth due to mutations in *his* structural genes. It detects frameshift or base-pair substitution mutagens that increase the frequency of His^+ revertants (Ames et al., 1975).

(2) Forward mutation tests - The forward mutation assay detects mutations in a larger portion of the DNA when compared to reversion assays. Any mutation that leads to an inactive gene product will be detected as compared to reversion systems where only the small number of mutational events that restore gene activity can be scored. One of the forward mutation tests used for detection of environmental chemicals is the *Salmonella typhimurium* L-arabinose assay. It uses strain SV3 which is sensitive to L-arabinose because of the *araD531* allele (Ruiz-Vasquez et al., 1978; Pueyo, 1978). Strains SV19, SV20, SV21 and SV50 were derived from SV3 (Pueyo, 1978; Whong et al.,1981) and carry additional mutations that increase their sensitivity. To compare the sensitivities of the arabinose forward mutation assay and the histidine reversion assay, a set of BA strains were developed from strain SV3 and from the histidine strains used in the Ames system (Ruiz-Rubio and Pueyo, 1982).

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(3) Growth inhibition tests - A number of DNA repair-deficient mutants have been isolated. These include the *polA* allele in *E. coli*, *recA* in *Bacillus subtilis* and *uvrB* in *S. typhimurium* which have been used to screen chemical mutagens. The extent of growth inhibition by the chemical is compared in wild-type and repair-deficient strains in these assays. The *polA* test in *E. coli* K-12 compares growth inhibition in the wild-type *E. coli* parent strain with that of the DNA polymerase I deficient mutant (Slater et al., 1971). A variety of carcinogens and mutagens have been detected by this test but those that require metabolic activation are not detected (Rosenkranz et al., 1976; Speck et al., 1978). The recombination assay uses *B. subtilis* M45 (Kada et al., 1972) which is a recombination-deficient strain.

(4) Tests using phage and DNA - Lambda prophage induction in *E. coli* K-12 is used as a screening method to detect potential mutagens and carcinogens. In this test the chemically induced lambda prophage produces plaques on a lawn of the host bacteria. These plaques are then counted after overnight incubation and compared with control values (Moreau et al., 1976).

In past years, a great degree of progress has been made in screening environmental mutagens and carcinogens, using the *Salmonella* mutation test first developed by Ames and his group (Ames et al., 1973; Ames et al., 1975). The histidine reversion assay of *S. typhimurium* uses a set of tester strains containing base-pair substitution and frameshift mutations. This assay has been widely used since Ames and his collaborators introduced the UV-sensitive (*uvrB*) and deep rough (*rfa*) alleles into the tester strains. The *uvrB* allele makes the cell more sensitive to many mutagens because of the lack of DNA excision repair and the *rfa* allele makes the cell wall defective in lipopolysaccharide structure which increases the cell permeability to chemicals. This assay detects mutations

that cause reversion or suppression to histidine prototrophy by specific mutational events (Ames et al., 1975; Levin et al., 1982).

Examples of *Salmonella* strains used in the *his* reversion assay are TA1535, used for detecting mutagens that cause base-pair substitutions and TA1538, which detects frameshift mutagens. Strains TA100 and TA98 respectively were developed from these standard strains by the addition of the R factor plasmid pKM101 (McCann et al., 1975). The presence of pKM101 enhances the sensitivity of the test and allows the detection of many additional mutagens (McCann et al., 1975). The exact mechanism by which pKM101 increases mutagenesis is not fully understood. McCann et al. (1975) proposed that pKM101 stimulated mutagenesis through recA-dependent repair. Walker (1977) and Mortelmans and Stocker (1976, 1979) suggested that the pKM101 enhances the cellular error-prone repair system. McPhee (1973, 1974) suggested that pKM101 could supply a mistake-prone DNA polymerase. It is now known that pKM101 contains two genes, *muc*A and *muc*B, which are analogues of the chromosomal *umu*C and *umu*D genes (Woodgate and Sedgwick, 1992). These genes are required for UV and some chemical mutagenesis (Bagg et al., 1981).

In general any mutational assay which detects specific reversion events has two important limitations. The assay may not detect mutagens which cause mutagenic lesions in DNA that cannot revert the point mutations in the strains being used in the assay. In addition to this, the reversion assay could overestimate the potency of mutagens: a large increase in the number of revertants might be induced in a strain because this mutagen specifically increases the one base-pair substitution or frameshift detected but causes no

other type of mutation (Ruiz-Rubio et al., 1984). Mohn et al. (1974), MacGregor and Sacks (1976), Ruiz-Vazquez et al. (1978), and Skopet et al. (1978) proposed forward mutation assays as the solution to these problems. Several mutagens have been detected by the forward mutation systems which were undetectable by the standard tester strains used in the His reversion assay (Pueyo, 1979; Sacks et al., 1982).

The L-arabinose assay of *S. typhimurium* is a forward mutation assay first developed by Ruiz-Vazquez et al. (1978) and subsequently characterized by Pueyo et al. (1979), Pueyo and Lopez-Barea (1979) and Whong et al. (1981).The arabinose operon (*araBAD*) is involved in using arabinose as an energy source and is located between the threonine and leucine genes (Englesberg et al., 1974). It is induced by arabinose and repressed when glucose is present (Englesberg and Wilcox, 1974). Its three structural genes, *araB*, *araA* and *araD*, encode the enzymes required to convert arabinose into xylulose 5-phosphate, an intermediate in the pentose phosphate pathway. Genes *araA*, *araB* and *araD* code for L-arabinose isomerase, L-ribulokinase and L-ribulose 5-phosphate 4-epimerase, respectively. The structural genes are contiguous and are transcribed into a polycistronic mRNA (Englesberg and Wilcox, 1974). The regulatory gene *araC* is involved in both positive and negative control of the *araBAD* genes.

The L-arabinose assay of *Salmonella* carries a mutation in *araD*, which blocks the utilization of L-arabinose as a carbon source. It also leads to the accumulation of a toxic intermediate, L-ribulose 5-phosphate, and thus inhibits bacterial growth when the L-arabinose operon is expressed (Englesberg et al., 1962). This mutagenic assay selects changes from L-arabinose sensitivity (Ara^S) to L-arabinose resistance (Ara^R). This

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phenotypic change is due to a forward mutation at any of the several genes in the L-arabinose operon (Pueyo and Lopez Barea, 1979).

The L-arabinose assay of *Salmonella* has been used to screen environmental mutagens. This assay selects for mutational events that change L-arabinose sensitivity to L-arabinose resistance (Ruiz-Vazquez et al., 1978). The tester strain SV3 cannot utilize L-arabinose and is sensitive to it. This strain is unable to grow in the presence of L-arabinose and another carbon source such as glycerol or casamino acids which is unable to repress the arabinose operon (Pueyo and Lopez-Barea, 1979). *S. typhimurium* strain SV3 is derived from *S. typhimurium* strain JL386 which is auxotrophic for tryptophan (*trp-294*), threonine (*thr-115*) and uracil (*pyr B92*) (Ruiz-Vazquez et al., 1978). A mutation, *araD531*, was induced by N-methyl-N-nitro-N-nitrosoguanidine in JL386 which was then designated strain SV3 (Ruiz-Vazques et al., 1978). *S. typhimurium* strains SV19, SV20 and SV21 were derived from strain SV3 by selecting for a *uvrB* deletion and a *rfa* mutation which arose spontaneously (Pueyo, 1978; Whong et al., 1981).

In most of the earlier microbial assays the detection of mutagens was very simple but was occassionally beset with experimental artifacts such as cell lysis, plating density and physiological changes that kill many cells, such as ultrasonic oscillation, lysosome exposure and osmotic shock (Auerbach, 1976; Grigg,1952). The frequency of arabinose-resistant mutants in strain SV3 remained constant after exposure to the above artifacts (Ruiz-Vazquez et al., 1978). This finding encouraged the use of the strain SV3 in the detection of mutagens. Similar results with strains SV19, SV20 and SV21 were demonstrated using SV3 as a control. It was also found that the properties of strain SV3 were not affected by the presence of the additional mutations *uvrB* or *rfa* or both (Pueyo,

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1978). In fact strains SV19, SV20 and SV21 showed increased sensitivity to mutagenicity by ICR-191 and 2-nitrofluorene compared to SV3 (Pueyo, 1978).

Whong et al. (1981) developed another strain, SV50, by transferring plasmid pKM101 into SV21, one of the tester strains derived from SV3 by Pueyo (1978). The mutagenic sensitivity of the Ara^R assay using SV50 was evaluated with 26 compounds both by plate incorporation and preincubation tests. The results demonstrated that the Ara^R assay detected the same chemical mutagens as the Ames assay. It was also shown that the preincubation test would be a better test protocol than the plate test for the Ara^R assay with SV50 (Xu et al., 1984).

Strains having *his ara* double mutants, which consist of both the histidine reversion and arabinose forward mutation systems, have been used to compare the relative sensitivities of these two systems. This set is the BA strains. Strains BA1, BA2, BA3 and BA4 were developed from the arabinose sensitive strain SV3 by phage P22 *int* transduction of the *araD531* allele into histidine strains *hisG46*, *hisD3052*, TA1950 (*hisG46*, *uvrB*) and TA1534 (*hisD3052*, *uvrB*), respectively (Ames et al., 1975). Strains BA5, BA6, BA7 and BA8 are the *rfa* derivatives of strains BA1, BA2, BA3 and BA4, respectively (Ames et al., 1973; Pueyo, 1978). Strains BA9 and BA10 are derived from strains BA7 and BA8 by the transfer of plasmid pKM101 from strain TA2000 (*pur* F145/pKM101) as described by McCann et al., (1975). Strain BA13, also derived from strain SV3, carries the mutant alleles *araD531*, *hisG46* and *uvrB* and pKM101 (Ruiz-Rubio et al., 1985).

Studies have shown that strains BA1 and BA2 with the genetic background of *hisG46* and *hisD3052* ,respectively, grow much better than the parental strain SV3 and

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the same time maintain sensitivity to L-arabinose (Ruiz-Rubio and Pueyo, 1982). It was also observed that the strain BA2 and its corresponding deep-rough derivative BA6 showed an increased frequency of spontaneous arabinose-resistant mutants when compared to strain BA1 and its derivative BA5. The reason for this higher mutation frequency was attributed to the genetic background of strain *hisD3052* when compared to strain *hisG46* (Ruiz-Rubio and Pueyo, 1982). It was seen that the increased growth rates in strains BA1 and BA2 compared with strain SV3 enhances the sensitivity of the arabinose system (Ruiz-Ribio and Pueyo, 1982).

BA strains 3, 7, 8 and 9 were utilized to make a quantitative comparison of the arabinose forward and histidine reverse mutation assays. These strains were exposed to 18 chemicals of different structural groups which were found to be mutagenic according to the histidine and arabinose assays of these strains. The results obtained from the histidine assay coincided quantitatively with those previously reported in the ames *Salmonella* strains (McCann et al., 1975; Neudecker et al., 1981). It was concluded that the new BA strains were comparable to the ames *Salmonella* strains in carrying out the histidine assay (Ruiz-Rubio et al., 1984). The chemicals tested in the arabinose assay with the BA strains gave results which showed higher levels of induced mutagenesis than with *Salmonella* TA100 (Ruiz-Rubio et al., 1984). The study also showed an increase in the accuracy with a liquid test when compared to the plate test with the Ara assay (Ruiz-Rubio et al., 1984). Thus the BA strains allow the utilization of both mutation assays which reduces the cost, time and the quantity of the sample to be tested in large-scale tests (Ruiz-Rubio et al., 1984). Ruiz-Rubio et al. (1984) based on their results, suggested that the His reversion assay can be replaced by the Ara mutation assay.

Ruiz-Rubio et al. (1985) tested chemicals which act preferentially at AT base pairs which have been previously tested with the *Salmonella* reversion strain TA102 (Levin et al., 1982). The arabinose forward mutation assay detected these oxidative chemical mutagens with increased sensitivity compared to the His reversion assay (Ruiz-Rubio et al., 1985). Based on their results, Ruiz-Rubio et al. (1985) have concluded that the forward mutation assay could be used for testing the mutagenicity of chemicals and it could replace the set of tester strains used in the His reversion assay.

Roldan-Arjona et al. (1989) have developed a simple method to test the lethal effects of chemicals. This test utilizes two strains of *S. typhimurium* (BA13 and BAL13) which are nearly isogenic. The strain BA13 (*araD531, hisG46, uvrB* and carries pKM101) is referred to as a 'mutation indicator' strain and strain BAL13 (*leu-6, uvrB* and carries pKM101) as a 'survival indicator' strain. In this study the selective plates showed colonies which were L-arabinose resistant mutants (Ara^R) derived from BA13 whereas the survival plates had BAL13 colonies which were histidine prototrophs and could utilize L-arabinose (Roldan-Arjona et al., 1989). Roldan-Arjona et al. (1989) have also reported that strains BA13 and BAL13 showed equal sensitivity towards the lethal effects of mutagens and BAL13 could be used to measure lethality for BA13.

Several studies had been carried out with the *S. typhimurium* His reversion mutation assay to make a quantitative comparison of carcinogenic potency in rodents and mutagenic potency in bacteria (Piegorsch and Hoel, 1988). These results showed a moderate correlation ($r= 0.4$) while using a variety of different chemicals (Piegorsch and Hoel, 1988). Roldan-Arjona et al. (1990) have examined monofunctional alkylating agents to show a quantitative relationship between mutagenicity in the arabinose forward mutation

test of *S. typhimurium* and carcinogenicity in rodents. There results have shown a highly significant correlation (r= 0.86, P< 0.01) which indicates that the Ara^R forward mutation test has the capability to predict the mutagenic potency of animal carcinogens (Roldan-Arjona et al., 1990).

Hera and Pueyo (1986) have studied the L-arabinose resistance test under different experimental conditions to optimize the use of this test for mass screening of mutagens. They have compared mutagenesis protocols using plate incorporation, the pre-incubation and the liquid test. Hera and Pueyo (1986) concluded that the liquid test is the most sensitive protocol for mutagenesis and in the arabinose forward mutation test the mutagenicity expressed as mutants per survivor is appropriate, especially when Ara^R selective plates are not supplemented with D-glucose (Pueyo and Ruiz-Rubio, 1984). It was suggested that the preincorporation and preincubation mutagenesis protocols could be utilized as alternative procedures especially in case of negative results from the liquid test. In addition, the use of a single tester strain instead of several histidine requiring mutants (Maron and Ames, 1983) is more advantageous for screening of mutagens (Hera and Pueyo, 1986).

Most mutagenesis tests have been conducted under aerobic conditions. Potential human carcinogens and mutagens are present inside the body, some parts of which may be fully anaerobic or partially anaerobic. The *Salmonella*/microsome test has been developed to screen mutagens which utilize a preparation of liver homogenate directly on the petri plates thus incorporating an aspect of mammalian metabolism into the test (Ames et al., 1975). Although the microsomal activation test includes an aspect of mammalian metabolism, the environment of this test is still aerobic. Oxygen free radicals

are generated during normal metabolism and are considered to be a potentially important class of endogenous mutagens/carcinogens (Totter, 1980; Ames, 1982, 1983). Therefore it becomes more important to test the mutagenicity/carcinogenicity of chemicals under contrasting anaerobic conditions. The L-arabinose resistance test of *Salmonella* is a highly sensitive test system which can be used for screening mutagens under anaerobic conditions.

Fluctuation tests under aerobic and anaerobic conditions were performed to detect mutagenic and genotoxic activity in human feces by Venitt and Bosworth (1986). Their studies have revealed that under aerobic conditions five out of six samples from the same donor were mutagenic with *S. typhimurium* TA100 and all six were mutagenic with *E.coli* WP2*uvrA*(pKM101). Under anaerobic conditions none of the samples were mutagenic with *S. typhimurium* TA100 but all six were positive with *E. coli* WP2*uvrA*(pKM101). The SOS chromotest (Venitt and Bosworth, 1986) gave negative results with all the samples. Venitt and Bosworth (1986) concluded that the donor excreted feces contained two different kinds of directly acting mutagens which could not be detected by the SOS chromotest even at high doses of the feces extract.

Venitt and Bosworth (1988) have also studied the mutagenicity of synthetic fecapentaene-12 under anaerobic conditions. Fecapentaenes are labile reactive compounds which are formed by the bacterial species of the colon in an anaerobic environment (Van Tassel et al., 1982a, 1982b). Fecapentaenes cause mutations by two different modes. They act as a base-pair substitution mutagen and also as a frameshift mutagen (Venitt and Bosworth, 1988). Studies have also shown that under anaerobic conditions mutagenic base-pair substitution activity of fecapentaenes was suppressed, whereas the frameshift

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activity was not; in fact it seemed to be enhanced (Venitt and Bosworth, 1988). Curren et al. (1987) have shown that fecapentaenes as base-pair substitution mutagens are more mutagenic than as frameshift mutagens. Therefore it was suggested that the anaerobic environment of the lumen protects against "reactive but labile compounds like fecapentaenes" which may become reactive and cause neoplasia in the oxygenated environment of the colonic epithelium (Venitt and Bosworth, 1988). However interpretation of these results is difficult due to inhibitor formation.

Materials and Methods

Bacterial strain BA13

Salmonella typhimurium BA13 (*araD531, hisG46, uvrB,* pKM101) was
obtained from Dr. Kristien Mortelmans and used for all experiments in this study. Strain
BA13 has been derived from strain BA3 by the addition of pKM101 (Ruiz-Rubio and
Pueyo, 1982). *S. typhimurium* BA3 was constructed by transduction with phage P22 *int*
carrying the *araD531* allele from strain SV3 which is arabinose sensitive into strain TA1950
(*hisG46, uvrB*) (Ruiz-Rubio and Pueyo, 1982). The R-plasmid pKM101 (McCann et al.,
1975) was introduced into BA3 by conjugation to obtain strain BA13 (Ruiz-Rubio et al.,
1985).

Media

Arabinose-resistant mutants (Ara^R) were selected on tryptone plates
containing 1% tryptone and 0.5% NaCl solidified with 1.5% agar and supplemented with
20% L-arabinose. Total cells were titered on tryptone plates without arabinose. Soft top
agar contained 0.6% NaCl and 0.7% agar. Liquid cultures were grown in L-broth which
consisted of 1% tryptone, 0.5% yeast extract and 0.5% saline. M9 salts (10x) contained
1% NH4Cl, 6% Na2HPO4, 3% KH2PO4 and 0.5% NaCl. Media for aerobic experiments
was sterilized by autoclaving for 15 minutes except for the 20% L-arabinose which was
filter sterilized.

For anaerobic experiments tryptone medium was autoclaved at 121°C for 30 minutes in 500ml quantities and was immediately transferred to the anaerobic chamber through a pass box. The media plates were poured in a second anaerobic chamber and kept in the chamber for one to two weeks before inoculating. For anaerobic experiments L-broth was autoclaved in 5ml tubes and transferred to the anaerobic chamber and kept for one week before use.

Mutagenesis Procedures

The *S. typhimurium* strain BA13 was grown to saturation both anaerobically (chamber) and aerobically (bench) for 48-72 h and diluted appropriately. For aerobic experiments 0.1ml of the cell suspension of bacteria were taken from the overnight BA13 cultures and together with 0.1ml of M9 salts (1x) and 3 microliters of EMS (3.75 mg/ml), added to a sterile test tube. Spontaneous mutation frequencies were determined by omitting the EMS. The mixtures were incubated at 37°C for 20 minutes. For selection of Ara^R mutants 2ml of molten soft top agar supplemented with 353 microliters of 20% arabinose, was added to the above tubes. The contents were gently mixed and poured evenly onto the surface of tryptone plates. The top agar was left to harden and the plates were then incubated for 4-5 days at 37°C. The mutant BA13 colonies appeared on the tryptone plates and were counted. The total number of cells was determined by placing 0.1 ml of the diluted overnight culture and 0.1ml of M9 salts, into a sterile test-tube and incubating at 37°C for 20 minutes. Two ml of molten soft top agar were added to the tube, gently mixed and poured onto the surface of tryptone plates and incubated for 4-5 days as described previously. For each experiment the number of mutant cells/ml or total cells/ml was averaged from the counts of two plates. A total of 12 experiments were conducted

under aerobic and anaerobic conditions. Similar procedures were followed under anaerobic conditions and the molten top agar was kept inside the chamber overnight. It was previously shown that EMS gave 6400 arabinose resistant mutants/10^8 viable cells with a dose of 56,395 nmol/ml (7,500 ug/ul) and 23% survival in the liquid test (Ruiz-Rubio et al., 1984). Based upon these previous results, concentrations of EMS were chosen that would be expected to give high numbers of mutants with low levels of killing.

Anaerobic chamber

The anaerobic chamber (Anaerobe Systems, San Jose, CA) consists of an air-tight acrylic chamber 152.4 cm L x 76 cm W x 44.7 cm H (Figure I). The armport system permits access to the interior of the chamber which consists of three parts: (a) sleeve assemblies which consist of a heavy duty rubber sleeve, a plastic cuff ring and a soft rubber arm cuff (the rubber cuff is responsible for making a snug, seal around the arm while working in the chamber without gloves); (b) armport doors which are used to seal the armports while the chamber is in active use; and (c) foot pedals which operate valves, one for gas and the other for vacuum, are used to make the sleeve area anaerobic before removing the armport doors to insert the arms into the chamber. A transfer module for moving materials in and out of the chamber and an incubator kept at 37°C are attached to either side of the chamber. The catalyst basket consists of aluminum pellets coated with palladium which are present to remove trace amounts of oxygen. The catalyst is reactivated by heating at 160°C for two hours. A positive pressure of ca. 7.6 cm of water is provided by an electronic pressure controller to maintain the gaseous environment of the chamber by the use of a displacement balloon to physically expel air from the chamber. Then the

chamber is refilled with a gas mixture of 10% hydrogen (H_2), 5% carbon dioxide (CO_2) and 85% nitrogen (Liquid Carbonic Corp. San Carlos, CA.).
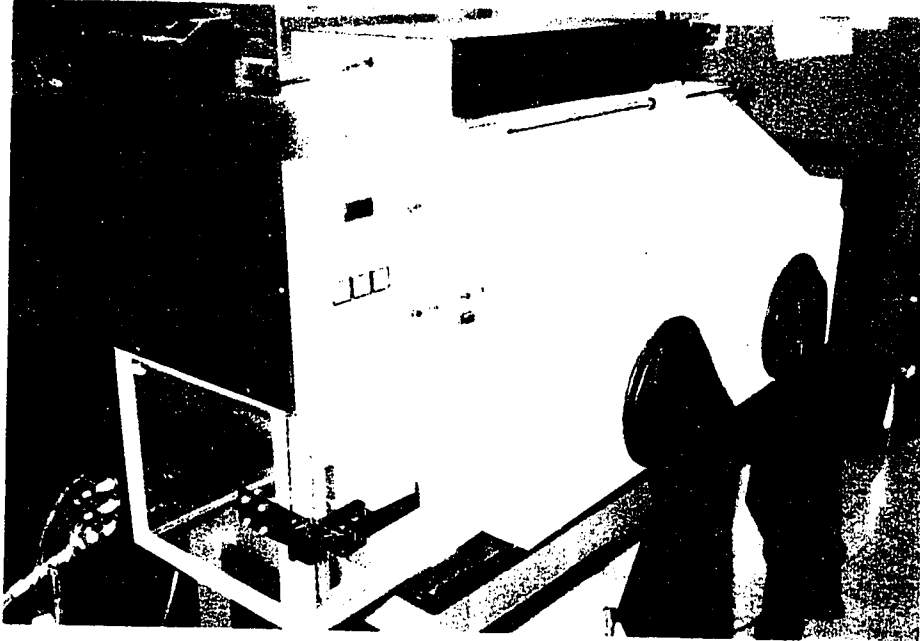
Figure 1
Anaerobic chamber, fitted with rubber gloveless sleeves in front and a transfer module on the left with control panel above. To the right of the chamber is an incubator which is accessible internally.

Results

The number of Ara^R mutants and survivors was determined by treating strain BA13 with a wide range of doses of EMS (0-7.5mg/ml). Over this range cell killing by EMS was insignificant (data not shown). Figures II and III show the induction of mutants by EMS in aerobic and anaerobic environments. Based on these results 3.75 mg/ml of EMS was used to induce mutations in subsequent experiments under both aerobic and anaerobic conditions.

Table I records the induction of Ara^R mutants in strain BA13 with EMS under aerobic conditions. Under aerobic conditions there is a 18-fold increase in the average induced Ara^R mutant frequency over the average spontaneous level. This might be an underestimation since experiments 4 and 5 show low levels of induced mutagenesis compared to the other experiments. Table II records the induction of Ara^R mutants induced with EMS under anaerobic conditions. Under anaerobic conditions there is a 55-fold increase in the average induced Ara^R mutant frequency over the average spontaneous mutant frequency. As shown in Table I and II the spontaneous mutant frequency is 15-fold higher under aerobic conditions than the average anaerobic frequency. Similarly the average induced (EMS) mutant frequency shows a 5-fold increase under aerobic conditions when compared to anaerobic conditions.
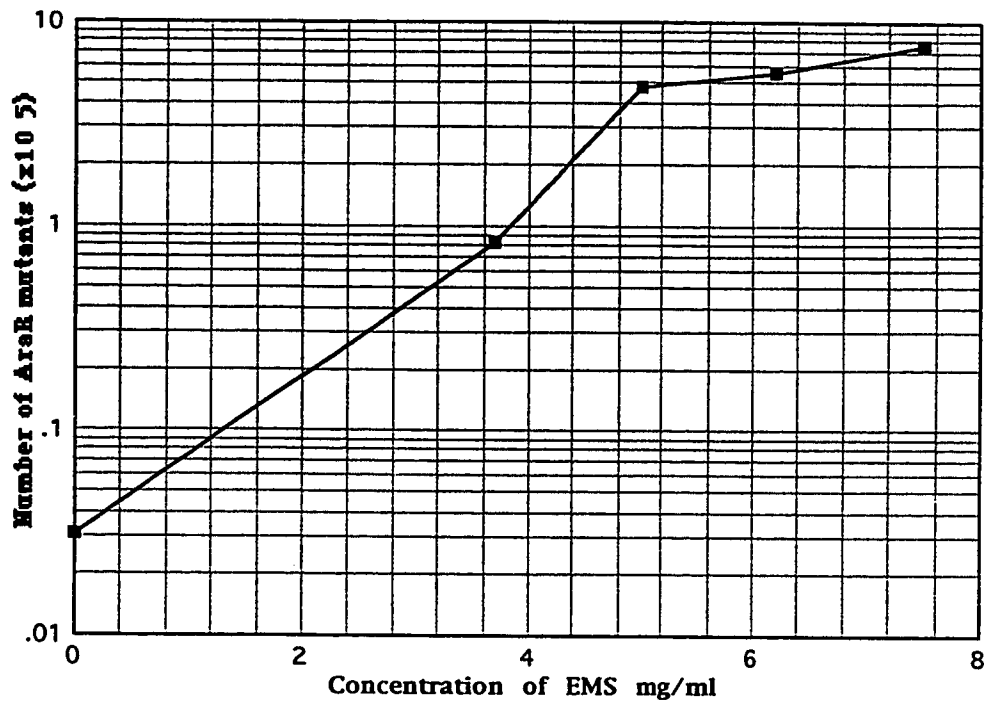
Figure II
The induction of Ara^R mutations in *S. typhimurium* by EMS under aerobic conditions.
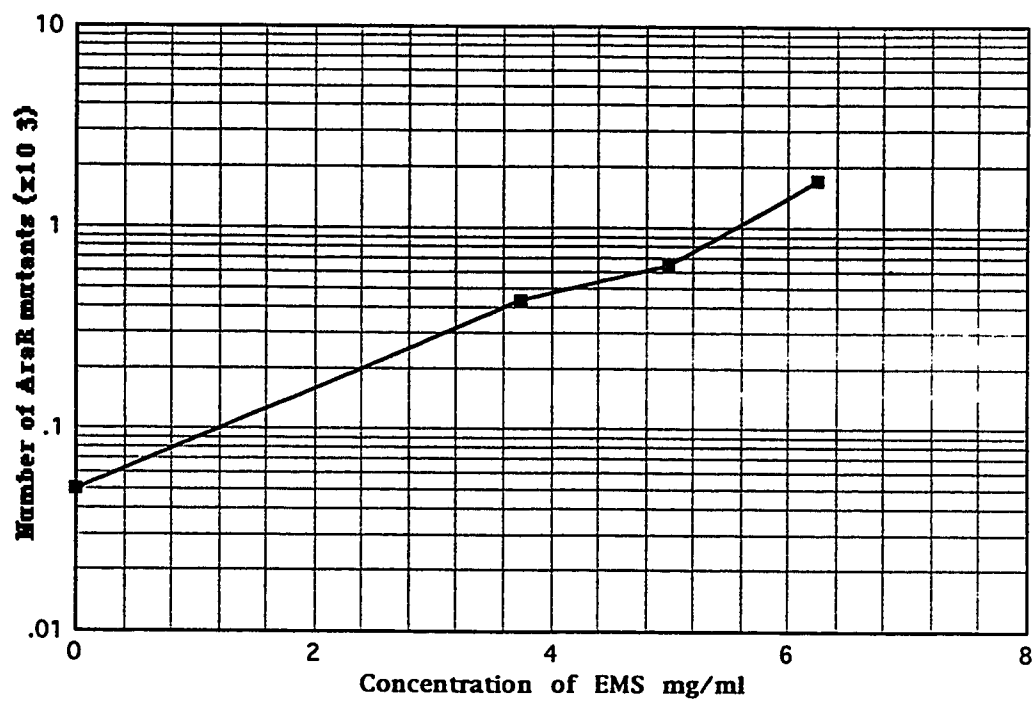
Figure III

The induction of Ara^R mutations in *S. typhimurium* by EMS under anaerobic conditions.

Table I

Table I

Induction of Ara^R mutations by EMS under aerobic conditions in *S. typhimurium* BA13

Experiment	EMS Dose	Mutant Cells/ml	Total Cells/ml (x 10^6)	Mutant Frequency	EMS Freq/ Spont Freq
1	0 mg/ml	178 x 10^2	630	2.8 x 10^{-5}	75
	3.75 mg/ml	131 x 10^{4a}		2.1 x 10^{-3}	
2	0 mg/ml	5 x 10^4	230	2.2 x 10^{-4}	14
	3.75 mg/ml	68 x 10^4		3.0 x 10^{-3}	
3	0 mg/ml	78 x 10^2	272	2.9 x 10^{-5}	38
	3.75 mg/ml	307 x 10^3		1.1 x 10^{-3}	
4	0 mg/ml	29 x 10^2	86	3.4 x 10^{-5}	8
	3.75 mg/ml	227 x 10^2		2.6 x 10^{-4}	
5	0 mg/ml	56 x 10^2	118	4.7 x 10^{-5}	3
	3.75 mg/ml	152 x 10^2		1.3 x 10^{-4}	
Average	0 mg/ml			7.2 x 10^{-5}	18
	3.75 mg/ml			1.3 x 10^{-3}	
SD^b	0 mg/ml			4.1 x 10^{-5}	
	3.75 mg/ml			1.1 x 10^{-3}	

a Not corrected for spontaneous mutations
b SD = standard deviation

Table II

Induction of Ara^R mutations by EMS under anaerobic conditions in *S. typhimurium* BA13.

Experiment	EMS Dose	Mutant cells/ml	Total cells/ml $(\times 10^6)$	Mutation Frequency	EMS Freq/Spont Freq
1	0 mg/ml	15×10^2	258	5.8×10^{-6}	78
	3.75 mg/ml	116×10^{3a}		4.5×10^{-4}	
2	0 mg/ml	11×10^2	190	5.8×10^{-6}	67
	3.75 mg/ml	74×10^3		3.9×10^{-4}	
3	0 mg/ml	9×10^2	277	3.2×10^{-6}	19
	3.75 mg/ml	171×10^2		6.2×10^{-5}	
4	0 mg/ml	11×10^2	227	4.8×10^{-6}	85
	3.75 mg/ml	92×10^3		4.1×10^{-4}	
5	0 mg/ml	18×10^2	350	5.1×10^{-6}	27
	3.75 mg/ml	49×10^3		1.4×10^{-4}	
6	0 mg/ml	14×10^2	346	4.0×10^{-6}	55
	3.75 mg/ml	77×10^3		2.2×10^{-4}	
7	0 mg/ml	9×10^2	231	3.9×10^{-6}	44
	3.75 mg/ml	40×10^3		1.7×10^{-4}	
Average	0 mg/ml			4.7×10^{-6}	55
	3.75 mg/ml			2.6×10^{-4}	
SD[b]	0 mg/ml			0.92×10^{-6}	
	3.75 mg/ml			1.4×10^{-4}	

a Not corrected for spontaneous mutations
b SD = standard deviation

Discussion

The present study evaluated the use of the Ara^R forward mutation assay with *S. typhimurium* BA 13 as a short-term test of chemicals for their mutagenic potency under anaerobic conditions. The anaerobic experiments were carefully performed in an anaerobic chamber to prevent exposure of cells to oxygen. Atmospheric oxygen levels in the chamber were monitored by indicator strips throughout the experiments and they did not reach detectable levels. However, it is possible that very low oxygen levels might have been present in the chamber atmosphere or in the bacterial media.

Spontaneous and induced mutation frequencies to L-arabinose resistance in the BA 13 strain of *S. typhimurium* were measured and compared under aerobic and anaerobic conditions. These mutations could have occurred in any of three genes of the *araBAD* operon (Pueyo and Lopez-Barea, 1979). Spontaneous mutation frequencies to Ara^R were reduced an average of 15-fold in the anaerobic environment compared to aerobic values (Table I and II). This suggests that the absence of oxygen is responsible for the reduction in spontaneous generation of L-arabinose resistant mutants.

These data indicate that over 90% of the aerobic spontaneous mutations to Ara^R are oxygen-dependent and suggest that oxygen is an important endogenous mutagen. Oxygen-induced mutations are usually thought to occur via the toxic by-products of aerobic respiration such as superoxide anion, hydrogen peroxide and hydroxyl radical (Fridovich,

1978) that cause oxidative damage to DNA and ultimately mutations (Storz et al., 1987; Greenberg and Demple, 1988).

The Ara^R assay is a forward mutation assay system which can detect different types of gene mutations (Xu et al., 1984). The tester strain used, BA13, carries a mutation in the araD gene (araD531) and is unable to convert L-ribulose 5-phosphate to D-xylulose 5-phosphate in the arabinose metabolic pathway (Pueyo and Lopez-Barea, 1979). This results in an accumulation of L-ribulose phosphate which is apparently toxic and leads to cell death. Therefore BA13 is sensitive to L-arabinose. However, forward mutations occurring in one of three genes (araA, araB, and araC) of the arabinose operon whose products act prior to the araD gene product in the arabinose catabolic pathway can lead to arabinose resistance (Pueyo and Lopez-Barea, 1979). Arabinose resistance can also be obtained through reversion events at the araD locus but these events are considered to be infrequent compared to the forward mutations to arabinose resistance (Pueyo and Ruiz-Rubio, 1984).

The Ara^R assay has been found to be responsive to a wide variety of chemical base-pair substitution and frameshift mutagens (Xu et al., 1984). Unfortunately none of the spontaneous or induced mutations to Ara^R from previous studies have been sequenced and mutational spectra are unknown. Therefore it is not possible to identify which particular spontaneous mutational events are oxygen-dependent. Previous studies have indicated that anaerobic conditions suppress the occurrence of some spontaneous base-pair substitutions but not of frameshifts (Venitt and Bosworth, 1988).

EMS was clearly mutagenic under both aerobic and anaerobic conditions in this study. However, it was about 5 times more mutagenic aerobically than anaerobically (Tables I and II). EMS is an alkylating agent that adds an ethyl group to several positions on bases in DNA. The most important premutational lesion seems to be the addition of an ethyl group to the O^6 position of guanine. This may lead to mispairing between guanine and thymine and subsequent GC -> AT transitions (Coloundre and Miller, 1977; Burns et al., 1986). It is not expected that oxygen would affect the actual alkylating event. However, the presence or absence of oxygen could affect a number of other processes that influence the final frequency of Ara^R mutations such as DNA repair that prevents the EMS-induced premutational lesions from being fixed as mutations or transport processes that affect the amount of EMS within the cell. Possibly some classes of Ara^R mutants are unable to form viable colonies in the anaerobic environment. These explanations for the higher EMS-induced aerobic Ara^R mutation frequencies compared to anaerobic values are neither mutually exclusive nor easily tested.

EMS has been previously detected aerobically as a potent chemical mutagen with the Ara^R assay system using both the preincubation test (as used in this study) and the plate incorporation test (Xu et al., 1984). EMS has also been shown to be mutagenic in an anaerobic environment using the TA100 strain of the Salmonella/Ames reversion assay which detects His$^+$ reversion events (Venitt and Bosworth, 1988). However, the situation is complicated with the Ames Salmonella tester strains since some authors have reported spontaneous and chemical-induced mutagenesis in an anaerobic environment (Hartman et al., 1984; MacPhee and Jolly, 1985; Venitt and Bosworth, 1988) while others have reported either the lack of mutagenesis or inability to measure mutagenicity (Droffner and Yamamoto, 1983; Mortelmans and Cox, 1992).

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Mortelmans and Cox (1992) examined spontaneous and chemical-induced mutagenesis with eight different *Salmonella* tester strains in a carefully defined anaerobic environment using prereduced media. No spontaneous or induced His^+ revertant colonies were observed for any of the strains. They also found no Trp^+ revertants with an *E. coli* WP2 (*uvrA*) tryptophan-dependent strain. The authors postulated that a nonspecific growth inhibitor is synthesized by stationary phase bacteria in a strict anaerobic environment that interferes with the growth of His^+ revertants cells. Small amounts of oxygen can interfere with the inhibitor activity and allow growth to occur. Mortelmans and Cox (1992) attributed the presence of low oxygen concentrations in limited anaerobic environments for the positive results with the Ames tester strains published by others. It is important to note that the presence of a growth inhibition masks the ability to directly measure anaerobic reversion frequencies. Reversion changes in the DNA are unable to be observed phenotypically by the visible presence of a His^+ colony.

The main purpose of this study was to evaluate the possible use of the Ara^R forward mutation system for mutagen testing in an anaerobic environment. Anaerobic spontaneous Ara^R mutation frequencies were readily measured as were the enhanced frequencies of a well-characterized chemical mutagen, EMS. Since both spontaneous and induced anaerobic mutation frequencies were significantly lower than aerobic values in this and other studies described above, a forward mutation system with its higher frequencies may be more useful than a reversion system where anaerobic frequencies may be too low to measure accurately. No evidence was found in this study for a growth inhibitor of the type described by Mortelmans and Cox (1992).

Anaerobic test systems for chemical mutagens are necessary. Many human cancers begin, at least in part, by somatic mutations occurring in tissue that is fully or partially anaerobic (Venitt, 1982). Some of these mutations may be caused by chemical mutagens as diverse as compounds synthesized by gut bacteria. Some chemicals may show different mutagenic specificities in an anaerobic environment compared to an aerobic one (Venitt and Bosworth, 1988). This study is the first step towards characterizing the Ara^R forward mutation assay as an anaerobic test system.

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