ADAPTATION TO ALKYLATION MUTAGENESIS IN *ESCHERICHIA COLI*

by

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ABSTRACT

Replicate isogenic populations of *E. coli* were propagated and maintained for over 4000 generations in order to investigate the adaptation of *E. coli* to increased levels of the mutagen methanesulfonic acid ethyl ester (EMS). Control "C" cell lines were propagated through daily serial culture in the absense of any mutagenic treatment. EMS adapted cell lines "E"/"e" were propagated through daily serial culturing and treated daily with 25μl of EMS following serial dilution. Mutation frequency and survival assays conducted in this investigation strongly suggest that prior long-term low dose exposure to EMS results in significantly higher levels of resistance to the lethal and mutagenic effects of larger challenge doses of EMS relative to long-term evolved control cell lines "C". In addition, both survival and inhibition disk assays suggest a cross adaptive response between EMS and MNNG, showing enhanced survival and reduced growth inhibition zones in cells adapted to EMS and challenged with MNNG. Preliminary competition experiments suggest relative fitness for the EMS adapted cell lines ("E"/"e") compared to the "C" control cell lines in both the presence of EMS. Unexpectedly the fitness estimates also suggest a higher relative fitness for the "E"/"e" EMS adapted cell lines in the absence of EMS treatment, suggesting that the EMS specific adaptation may also result in improved fitness in novel environments. Despite the adaptive advantage for the "E"/"e" cell lines suggested by the fitness estimates, the results from the competition experiments are insignificant due to the high degree of variability among replicate fitness estimates. Attempts to induce the adaptive response repair pathway were not successful in either the control "C" or the EMS adapted "E"/"e" cell lines suggesting that enhanced resistance seen in the

adapted "E"/"e" cell lines could likely be a result of enhanced activity of the constitutive transferase Ogt and the constitutive glycosylase Tag. The *ada* and the *ogt* genes encode the induced and the constitutively-active DNA methyl transeferases in *E. coli.* As such they appeared to be the most likely candidates for genetic changes responsible for the enhanced resistance to the lethal and mutagenic effects of large doses of alkylating agents in the long-term EMS adapted "E"/"e" cell line. However, the DNA sequences analyzed for the *ogt* and the *ada* genes for both the long-term evolved control *E. coli* cell line "C" and the long-term-evolved EMS adapted "E'/"e" cell line indicate no sequences differences between these two cell lines. Previous studies have primarily observed *E. coli's* ability to phenotypically acclimate over very short time intervals to EMS. This analysis has shown that long-term genetic adaptation to low doses of EMS results in enhanced resistance to both the lethal and mutagenic effects of larger challenge doses of EMS.

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Chapter 1

General introduction

1.1 Selection and adaptation in microorganisms

Two of the key elements of biological evolution are the adaptation of organisms to their environment and the divergence of populations and species. Evolutionary biologists endeavour to elucidate the dynamics of these processes and to understand the contributions of natural selection, chance events and historical constraints (Lenski *et al.* 1991). A wide range of evolutionary questions have been addressed using long-term selection experiments with microbial populations (Elena and Lenski 1997; Korona 1996). Bacteria such as *Escherichia coli* have proven to be useful model systems due to their short generation times and large effective population sizes. *E. coli* has been extensively used in microbial genetics and molecular biology and therefore many of its genetic components and interactions are well characterized. Isogenic

populations can be initiated and maintained for hundreds of generations in strictly controlled environments (Travisano *et al.* 1995; Dykhuizen and Hartl 1983) and propagated with relative ease allowing intensive replication of experiments which is often necessary in order to observe minute effects (Elena and Lenski 1997b).

1.1.1 Selection in chemostats

Laboratory studies of evolution using microorganisms can be conducted in various environmental systems such as daily culturing in test tubes, on petrie plates, or in continuous culture devices know as chemostats (Dykhuizen 1993). In chemostats the division of microorganisms is continuous while populations are held constant and can be maintained for thousands of generations representing potentially evolving systems (Dykhuizen and Hartl 1983). The chemostat provides an environment that allows the experimenter to manipulate the system with rigorous controls and to test various relations (Dean 1989). In general the long-term evolutionary studies conducted in continuous culture devices have been classified into two distinct categories; the study of adaptive evolution and the study of natural selection. The former follows the adaptive evolutionary path of a population in a defined environment, while the latter characterizes the effects of changes in genetics or environment on selection coefficients (Dykhuizen 1993).

A vast amount of literature exploring evolutionary mechanisms has come about through the use of long-term chemostat studies. Dykhuizen and Hartl employed the use of chemostats in their series of studies on differential growth rates associated

with induced, naturally occurring or genetically induced mutations. The chemostat environment also enabled them to study the effects of plasmids and transposons on growth-rate, physiology and on the metabolism of cells in defined environmental conditions (Dykhuizen and Hartl 1983). One of the key observations within these series of studies was the presence of selective sweep events otherwise referred to as periodic selection. Long-term chemostat studies indicate that *E. coli* in lactose limited cultures undergo a predictable selective sweep for lactose constitutive cells within the first 100h. These events are quite common in long-term microbial cultures and occur quite predictably with defined physiological and genetic purpose (Dykhuizen and Hartl 1983; Elena, Cooper and Lenski 1996). The consistent results within independent serial cultures suggest that the genetic basis for periodic selection of lactose constitutive cells most likely involve the same loci (Dykhuizen and Hartl 1983; Dykhuizen, Dean and Hartl 1987). However, in environments with fluctuating lactose concentrations or in media containing no lactose, constitutive lactose mutants are selected against. This finding suggested strong selective forces aimed at sustaining control factors regulating gene products such as the lactose operon in natural populations (Dykhuizen, Dean and Hartl 1987). Selection for naturally occurring enzyme variants such as those observed with lactose have different physiological effects and in turn have strong effect on reproductive success. Another phenomenon quite pronounced in long-term evolving cultures accompanying these selective sweep events is a hitchhiking effect of all of the genes originally present in the selected clone (Dykhuizen and Hartl 1983). These findings prompted a series of studies on adaptive topography in order to provide an understanding of the mechanisms involved in the maintenance of various types of polymorphisms in natural populations. As a result

various metabolic models have been developed. Antony Dean (1989) initiated experiments as an extension to the experiments conducted by Dykhuzien and Hartl on the lactose operon in *E. coli.* The kinetics of β-galactosidases and permeases of six lactose operons in competing strains of *E. coli* suggests fitness proportional to lactose flux and as a result selection appears to favour permease mutants with high activity and consequently the control of galactosidase diffusion will reside entirely in the cell wall (Dean 1989). This model predicts that an increase in lactose would cause an increase shift in the control from the β-galactosidase to permease which would then in turn be exposed to intense selection. This reasoning implies that competition and selection may actually be intensified in environments with an abundance of resources and not necessarily maximized with a scarcity of resources as previously believed. These observations also demonstrate that alleles of different genes effecting the same trait may be exposed to different intensities of selection (Dean 1989).

Analysis of naturally occurring alloenzymes and their selection effects in temporally variable environments suggest that variation that is selectively neutral in one environment may be subject to intense selection in another environment due to changes in enzyme activities, flux and fitness (Dean 1994). Those polymorphisms exhibiting a latent potential for selection tend to be neutral in common environments and become strongly selected for in rare environments. Polymorphisms displaying kinetic variation, large control coefficients in metabolic pathways and have strong effects on fitness appear to have the greatest probability of being subjected to intense selection. In turn, those polymorphisms containing smaller control coefficients tend to be candidates for neutrality (Dean 1994). This may have a pronounced effect on

allele frequencies within a population and suggests that environmental variability may play an important role in maintaining polymorphisms at certain loci (Dean 1994). These chemostat studies have been useful to form metabolic models that have enhanced our understanding of the complex interactions between kinetic variation and the environment and the influence of these two factors on metabolism and fitness.

1.1.2 Adaptation in long-term microbial cultures

It is suggested that the key factor in the ability of microbial populations to survive diverse environments is their large populations sizes. Bacterial populations often face many environmental growth inhibiting challenges and as a consequence they have evolved survival mechanisms that enable them to cope and prevail against extreme challenges. Since these survival mechanisms are dependent upon the appropriate genetic variables it has been suggested that the organization of these genetic variables may be modulated by biochemical complexes that respond to physiological inputs and thereby serve an adaptive function (Lenski 1995). These biological complexes may be up-regulated during times of starvation or other stresses and as a result increase the mutation frequency and promote processes such as gene shuffling, duplication and dispersal of gene sequences or amplification of repetitive DNA. The combination of an increased mutation frequency and large population size of microorganisms allow rare beneficial mutations to occur that permit survival and further propagation of the resistant clones in the population. Studies indicate that often these genetic changes are biased towards adaptive mutations (Lenski 1995) leading to alternative metabolic pathways that are otherwise silenced or down regulated, or special pathways for en-

hanced repair or mutation (Koch 1993). These survival mechanisms may be purely incidental, while others may be a consequence of prior long-term evolution that act as a form of insurance implemented at the time of challenge with a minimal energy expenditure for maintenance (Koch 1993).

Over the past decade Richard Lenski and his collaborators have embarked upon a series of long-term evolutionary experiments to study divergence and adaptation of *E.coli.* In their experiments they have attempted to elucidate the evolutionary forces that promote either parallel changes or divergence in traits, as well as the mechanisms maintaining genetic variability within populations. These studies employed the use of natural selection techniques, whereby a variety of environments are imposed upon experimental populations of known genetic composition and evolutionary trajectories are followed over time (Lenski and Travisano 1994). Competition experiments are most commonly used to determine direct estimates of fitness of derived populations relative to their common ancestor in both selective and novel environments.

Upon introduction into novel environments replicate populations of *E.coli* show similar fitness trajectories, and undergo initial rapid increases in mean fitness followed by a plateau (Lenski *et al.* 1991; Lenski and Travisano 1994). The deceleration of fitness increases over the course of the experiment is a common feature in long-term evolutionary studies and suggests that intense selection pressure triggers the rapid fitness increase as a result of the initial change in environment (Lenski and Travisano 1994). The leveling off of fitness trajectories may be a consequence of an imposed limit to adaptive evolution due to metabolic constraints despite adaptive mutations directing metabolic activity to faster growth (Korona 1996). Another

suggestion has been a limitation on fitness increase due to a finite number of classes of beneficial mutations that eventually lead to a diminishing rate of increase in mean fitness (Lenski *et al.* 1991). These observations also imply that the evolutionary process may follow more of a predictable path rather than being as unpredictable and chaotic as often suggested by evolutionary biologists (Dykhuizen 1993).

In long-term evolving cultures there is generally a substantial increase in the fitness of the derived population relative to the ancestral population. In addition, significant variation in mean fitness is usually detected (Lenski *et al.* 1991; Lenski and Travisano 1994). Common features of these evolutionary experiments are: uniform maximum growth rates and similar mean fitness trajectories in the final clones. This suggests evolutionary parallelism among the replicate populations (Korona 1996; Lenski *et al.* 1991; Lenski and Travisano 1994). Although the mean fitness values in the evolved populations tend to be similar, it is not necessary for the adaptations involved to be identical. This is evident through the formation of alternative metabolic pathways in microorganisms exposed to identical environmental conditions. Experimental evidence suggests that the evolutionary process of adaptation of microorganisms to a simple constant environment may favour the formation of stable polymorphisms within an environment even in the absence of challenge in order to maximize growth rates. Populations of *E. coli* initiated from single clones develop polymorphisms that stably coexist and utilize different patterns of secretion and uptake of metabolites (Rosenzweig *et al.* 1994). Derived genotypes from long-term continuous glucose cultures diverge considerably from one another in their competitive fitness ability in novel environments containing limiting nutrients such as lactose and maltose. The

improvement in fitness and genetic fitness variance observed in glucose adapted cultures when grown in novel nutrient mediums appears to be correlated with different nutrient uptake mechanisms (Travisano 1997). Although these independent populations acquire similar adaptations to a particular environment these observations suggest that the genetic basis for the physiological adaptations may differ (Hall 1988; Lenski 1988).

Many studies have shown that there is often a cost associated with adaptation to a particular environment. This cost may be a consequence of an increased mutation rate which may increase the frequency of rare beneficial mutations as well as deleterious mutations. The second type of cost is a result of the specificity of adaptation, which may often lead to poor adaptation in other environments (Bell 1990). For example, resistance of *E.coli* B to T4 virus results in defects in the lipopolysaccharide (LPS) core of the cell envelope (Lenski 1988; Lenski 1988b). It has been suggested that selection of novel phenotypes is often associated with maladaptive pleiotropic effects, and as a consequence trade-offs are often exhibited between measures of fitness across environments (Lenski 1988). These maladaptive effects are observed early in the experimental lines and diminish with further adaptation due to compensatory substitutions (Lenski 1988b).

The above evolutionary studies have also been applied to the long-term study of genetic adaptation of microorganisms to temperature (Bennett, Dao and Lenski 1990; Bennett, Lenski and Mittler 1992; Bennett and Lenski 1993; Leroi, Bennett and Lenski 1994; Mongold, Bennett and Lenski 1996; Bennett and Lenski 1996). Temperature is an environmental factor of great significance due to its variability and its

influence on biological rate processes in many organisms (Bennett, Lenski and Mittler 1992). Consistent with the glucose studies the direct fitness response is greater under novel thermal conditions as opposed to the ancestral thermal regime. Populations demonstrate considerable adaptation to novel thermal regimes, which appears to be highly temperature specific (Bennett and Lenski 1993). Despite the specificity of the adaptation, fitness is not compromised in other thermal regimes, nor significant changes in thermal limits observed (Bennett and Lenski 1993). However, population lines continually subjected to varying thermal regimes coined "generalists", show an increase in fitness relative to their common ancestor over the widest rage of temperatures. These studies can be used to investigate and predict the response of natural populations to varying environmental conditions (Bennett, Dao and Lenski 1990).

1.2 Mutation

A mutation is a heritable change that may confer a genetic advantage, may be deleterious to an organism or may have no selective effect (Singer and Kusmierek 1982). Mutations may be induced by radiation or environmental chemicals but may also result as a consequence of the inherent instability of nucleic acids (Singer and Kusmierek 1982). Mutagenic efficiency in a particular system is dependent upon a variety of factors such as repair systems, metabolic activation or deactivation, confirmation and the effect of the mutagen on the physiology of the cell. Therefore, an effective mutagen in one system may prove to be relatively harmless in another (Singer and Kusmierek 1982). For example, N-methyl-N'nitro-N-nitrosoguanidine (MNNG) has

high levels of mutagenecity in intracellular λ phage, *Salmonella*, and *E. coli* and is a poor mutagen in T4 and S13 phages, *Haemophilus influenzae* DNA, and TMV RNA (Singer and Kusmierek 1982). The process of mutagenesis can be classified into three distinct modes: the mutagen may take the place of a normal base a base analogue, the mutagen although not incorporated alters the base(s) causing specific mispairing, or the mutagen damages base(s) thereby impairing specific base pairing (Miller 1983). There are a variety of mutagens in the environment that we are constantly exposed to giving added importance to the study of mutagenesis and its pathways.

1.2.1 Mutation Rates

A thorough understanding of evolutionary processes requires an understanding of both the nature of and the rates of spontaneous mutation (Hall 1988). The rates of spontaneous mutation can vary widely between and within organisms (Drake 1991). The average spontaneous mutation rate in *E. coli* has been estimated at $3x10^{-3}$ mutations per genome per generation (Drake 1991). The Lac system has proven to be a useful system for the study of mutational spectrum. Barry Hall (1988) utilized this system to demonstrate that mobile genetic elements often play a key role in the generation of adaptive mutations and that mutation rates are highly variable and may be under environmental modulation similar to other biological and cellular processes. Lac^- mutant *E. coli* cells when grown in a lactose medium alter the *ebg* operon for the catabolism of lactose and other β-galactosidase sugars. All *ebg* mutants capable of growing on lactose are found to carry two specific spontaneous mutations in the *ebgA* (structural) and the *ebgR* (regulatory) genes, occurred 10^8 times more frequently

than expected. Further analysis with more complex systems are consistent with these findings. A specific Lac construct requires two specific mutations in the *bglF* operon and an excision of IS103 from within *bglF* in order for the cells to utilize salicin. Within this construct these two mutations are found to occur again at frequencies much higher than expected, by a factor of $4X10^8$ and $2X10^8$ respectively. The increased frequency of IS103 excisions appears only at times when its excision would be selectively advantageous. Hall suggests that these observations are consistent with a modulation of mutation probability in specific loci during stressful conditions.

Mounting evidence suggests that the hypermutability observed by Hall (1988) may be localized to certain regions of the genome (Moxo and Thaler 1997). Pathogens have exhibited high mutation rates in a subset of genes that usually code for cellular regions involved in interactions with host molecules (Moxo and Thaler 1997). It is within these genes that most of the phenotypic variants are believed to be generated, and enable the cell to explore the host environment without significantly affecting its fitness (Moxo and Thaler 1997). Pairwise chemostat competition experiments demonstrate that bacterial strains that harbour mutator genes have a significant advantage over their otherwise isogenic counterparts that have lower mutation rates (Chao and Cox 1983; Chao *et al.* 1983). The transposable element Tn10 appears to act in a similar manner as mutator alleles in bacterial strains and confer a fitness advantage to the strains (Chao *et al.* 1983). The proposed manner in which mutator alleles work is through the increased generation of beneficial mutations. Although the mutants may reduce individual fitness, these mutant populations will tend to efficiently generate adaptive mutations and in turn produce and increased portion of

higher fitness mutants that are significantly superior to their parent clones by large fitness increments (Chao and Cox 1983; Taddie *et al.* 1997).

It has been suggested that asexual populations exposed to sporatic adaptive evolution will commonly exhibit a transient increase in mutator frequency (Taddie *et al.* 1997). It is often questioned why a mutator allele with such pronounced effects remains relatively rare. Taddei *et al.* (1997) suggest that a high reversion in the mutator alleles can eliminate linkage disequilibrium with favourable mutations. Simulations indicate a tendency for these mutator alleles to increase in frequency when: abundant and decrease in frequency when rare resulting in a stochastic mutation frequency patterns (Taddie *et al.* 1997). It has been suggested that selection may alter the mutation rates through forward and reverse mutations in the mutator alleles (Taddie *et al.* 1997). In clonal populations of *E. coli*, mutators appear to increase in frequency by hitch-hiking in association with an adaptive mutation (Sniegowski, Gerrish and Lenski 1997). This hitch-hiking effect will only occur if the cost associated with the mutator is not greater than the benefits of the mutations produced (Sniegowski, Gerrish and Lenski 1997).

The evolutionary adjustment of mutation rates is not confined to microorganisms. *Drosophila melanogaster* exhibit a fine-tuned adjustment of mutation rates in response to x-ray irradiation levels (Nothel 1987). An increased mutation rate and enhanced resistance to irradiation is observed when dosage is increased, while mutation rates and resistance remain constant when irradiation levels do not change. Resistance to irradiation in *Drosophila* appears to be controlled by a single genetic factor which is believed to modify or control mutator genes (Nothel 1987). The variability observed

in mutation rates among and within organisms appears to be controlled by selective forces, which aim to balance the costs imposed by deleterious mutations with the ability to adapt to a changing environment.

1.2.2 Alkylation damage

Organisms are continually exposed to a variety of natural mutagens within the environment and therefore have evolved mechanisms which protect against the toxicity and mutagenecity of these agents (Sedgwick and Vaughan 1991). A large amount of mutant sequence data has been made available through forward mutational studies in *E.coli* (Horsfall *et al.* 1990). This has expanded the available information on chemical specificity of mutagens, the influence of DNA sequence on mutation formation, and the efficiency of repair. A significant portion of powerful mutagens and carcinogens consist of alkylating agents (Horsfall *et al.* 1990). An example of a powerful alkylating agent is methanesulfonic acid ethyl ester (EMS). EMS belongs to a class of alkylating agents that react through an SN_1 mechanism and produce primarily O^6-alkylguanine lesion. SN_1 alkylating agents result in 25-30 times more O^6-alkylguanine lesions that their SN_2 alkylating counterparts MMS, DMS and MeI (Sledziewska-Gojska and Torzewska 1997). O^6-alkylguanine is a potentially mutagenic lesion causing mispairing with thymine during DNA synthesis (Horsfall *et al.* 1990). Studies indicate that this mispairing appears to be less stable that the non-mispaired counterparts and therefore it has been suggested that the mutagenicity of these lesions is probably due to misreading of the O^6-alkylguanine as adenine (Friedberg, Walker and Wolfram 1995). In many organisms the distribution of GC

to AT transitions is highly influenced by the context of DNA. For instance, alkylnitrosoguanidines and alkylnitrosoureas preferentially cause G:C to A:T transitions at guanines preceded by a purine residue. However the frequency and distribution of GC to AT transitions resulting from EMS alkylation damage does not exhibit 5′ flanking base influence or strand bias in *E. coli* (Horsfall *et al.* 1990).

Repair of alkylation damage

The integrity of genetic information contained in cellular DNA is highly dependent upon the effectiveness of the DNA repair systems in an organism (Lehmann and Karran 1981). As a result organisms have a variety of repair mechanisms to combat the damage often induced by radiation or environmental agents. In *E. coli* O^6-alkylguanine repair is mediated by two types of alkytransferases, the *ogt*, a constitutively expressed gene product (19-kDa) and the induced *ada* gene product (37-kDa) (Roldan-Arjona *et al.* 1994). The adaptive response mechanism was first observed in a study conducted by Samson and Carins in 1977. Upon continuous exposure of *E. coli* to low doses of the alkylating agent MNNG (N-methyl-N′nitro-N-nitrosoguanidine), *E. coli* cells developed significant resistance to the mutagenic and lethal effects of higher lethal challenge doses of MNNG. This inducible repair mechanism is distinct from UV-induced error-prone SOS repair since it is error-free allowing increased survival at no cost to the cell and a significantly reduced mutation frequency compared to non-adapted cells (Lindahl *et al.* 1988; Samson and Cairns 1977). Studies indicate this repair process to be independent of *recA* and *lexA* genes and not inducible by UV or 4-nitroquinoline 1-oxide (Jeggo *et al.* 1977; Schendel *et al.* 1978). In *E. coli* the inducible O^6-methylguanine-DNA methyltransferase I (O^6MGT I) or Ada

protein acts to reduce alkylation damage by transferring the methyl group from the alkylated DNA to its own cysteine residue, leaving behind an unmodified guanine base (Demple *et al.* 1982; Lindahl *et al.* 1988). The methylated Ada molecule is quite stable and not actively demethylated and as a result is inactivated and expended in each reaction (Friedberg, Walker and Wolfram 1995). Ada contains two distinct methyl acceptor sites, one for methylphosphotriesters and one for O^6-methylguanine in the N-terminal and the C-terminal regions of the protein respectively (Lindahl *et al.* 1988). Characterization of the Ada protein has revealed a dual role, acting as a regulatory protein in addition to a DNA transferase. Methylation of this protein converts it into a strong transcriptional activator for itself and three other genes *alkA*, *alkB*, and *aidB* involved in this inducible response (Sedgwick and Vaughan 1991; Vaughan *et al.* 1991). The Ada protein binds to a promoter region of the *ada/alkb* and *alka* genes. The binding sites of the *ada/alk* and *alk* genes contain a common promoter region (AAANNAAAGCGCA) termed the "Ada Box" (Lindahl *et al.* 1988; Nakamura *et al.* 1988; Landini and Volkert 1995; Friedberg, Walker and Wolfram 1995). Mutations in this sequence eliminate transcriptional activation of the *ada* gene (Nakamura *et al.* 1988; Landini and Volkert 1995). Pre-treatment of cells with SN_2 alkylating agents such as MMS or DMS results in the inhibition of O^6MGT I activity. This inhibition may be a consequence of self methylation of the Ada protein (Sledziewska-Gojska and Torzewska 1997) which has also been shown to be the main cause for inhibition of transcription activation in *Salmonella typhimurium* (Vaughan and Sedgwick 1991). Evidence suggests that the mismatch repair system compensates for the lack of transferase activity and excises the mismatches formed in DNA by O^6-methylguanine (Sledziewska-Gojska and Torzewska 1997). Although the adaptive

response mechanism reduces the mutagenic as well as the killing effects of alkylating agents, studies suggest that the mechanisms leading to killing resistance is different from that of mutagenic resistance (Jeggo 1980). This appears to be due to the type of alkylation damage incurred after exposure to large doses of alkylating agents. O^6-methylguanine is the primary mutagenic lesion caused by alkylation damage while 3-methyladenine and 3-methylguanine occur in significantly smaller amounts however these are the primary lethal lesions caused by alkylation damage (Friedberg, Walker and Wolfram 1995). Mutagenic adaptation requires the direct reversal of O^6-methylguanine base damage, while killing adaptation requires excision repair of both 3-methylguanine and 3-methyladenine and studies suggest that these two processes do not always occurr in parallel (Friedberg, Walker and Wolfram 1995). Killing adaptation is more readily induced requiring adaptive concentrations of alkylating agents insufficient to induce mutagenic adaptation (Jeggo 1980). Studies indicate that killing adaptation requires a functional DNA polymerase I, while *polA* mutants still maintain the ability to induce mutagenic adaptation (Karran, Hjelmgren and Lindahl 1982). It is DNA polymerase I that is believed to have a role in the gap filling process needed post excision repair (Karran, Hjelmgren and Lindahl 1982). Following alkylation damage, most of the 3-methyadenine adducts are removed by the constitutive DNA glycosylase I also known as the tag^+ gene product. However, this glycosylase is insufficient to remove all alkylation damage following a large dose alkylation exposure, and 3-methyladenine and 3-methylguanine remain in the minor groove of the helix which is lethal to cell unless excised. The methyl groups protrude into the minor groove of the DNA helix causing in interference in base stacking interactions and as a result blocking replication (Hickson 1997). This repair mechanism is

conducted by the inducible DNA glycosylase II. The *E. coli alka* gene is the structural gene for this enzyme also referred to as 3-methyl-adenine-DNA glycosylase (Lindahl *et al.* 1988; Landini and Volkert 1995; Friedberg, Walker and Wolfram 1995). This enzyme recognizes and excises a variety of methylated DNA bases (Lindahl *et al.* 1988; Landini and Volkert 1995). Like O^6-methylguanine-DNA methyltransferase I, *alkA* is controlled by the ada^+ locus and is considered a component of the adaptive response.

Other inducible adaptive response genes

There are three additional genes to date that play a role in the adaptive response of *E. coli*: *alkA*, *alkB* and *aidB*. The *alkB* gene is located downstream from the *ada* gene and encodes a 24-kDa protein of unknown function (Friedberg, Walker and Wolfram 1995). Due to its location the *ada* and *alkB* genes form an operon (Lindahl *et al.* 1988). *alkB* mutants do not effect the induction of either *alkA* or *ada*, therefore its function is more likely to be direct removal of mutagenic alkylation lesions rather than a regulatory role (Lindahl *et al.* 1988). Studies indicate that *aidB* encodes a protein homologous to the mammalian isovaleryl coenzymeA dehydrogenase (Landini and Volkert 1995) and has two mechanisms of induction. *aidB* induction can occur independent of Ada through oxygen-limiting conditions or by the addition of sodium acetate (Landini and Volkert 1995). This mechanism requires the presence of a functional *rpoS* gene, that encodes for a σ^s, an alternative sigma factor for RNA polymerase required for the expression of stationary phase-specific genes (Landini and Volkert 1995). The transcriptional activation of *aidB* through the adaptive response mechanism requires higher concentrations of methylated Ada than required for the

ada promoter. The *aidB* Ada-binding site shows a weak homology to the Ada box consensus sequences reported in previous papers (Nakamura *et al.* 1988). This could result in a lowered affinity for Ada to the *aidB* Ada-binding site (Landini and Volkert 1995). It has been suggested that this lowered affinity might indicate an emergency role for *aidB* that is induced in response to extreme levels of methylating damage (Landini and Volkert 1995).

Termination of the Adaptive Response

Since the methyltransferases involved in the adaptive response undergo irreversible inactivation, the response is limited to the number of available intra-cellular transferase molecules. Upon induction of the adaptive response, levels of Ada have shown to increase dramatically from approximately 1 molecule per cell up to 3000 molecules per cell (Cairns *et al.* 1981; Saget and Walker 1994). Levels above 200-500 unmethylated Ada molecules per cell appear to result in inhibition of *ada* transcription, which is not observed in cells containing identical levels of methylated Ada molecules (Saget and Walker 1994). These observations suggest that Ada may negatively modulate its expression. The proposed theory suggests that upon completion of repair, Ada transcription will continue producing increased levels of unmethylated Ada reaching inhibiting levels and resulting in decreased *ada* transcription (Saget and Walker 1994). Proteolytic degradation of the activated Ada protein is another mechanisms that has been suggested for termination of the adaptive response (Friedberg, Walker and Wolfram 1995).

O^6-methylguanine DNA methyltransferase II

The *ogt* gene product, O^6-methylguanine DNA methyl transferase II (O^6MGTII) is constitutively expressed and is responsible for repair of the majority of the alkylated lesions with a distinct preference for O^4-methylthymine, a product common to alkylating and bulky ethylating agents (Takano, Nakamura and Sekiguchi 1991; Friedberg, Walker and Wolfram 1995). It has been suggested that this repair mechanism is limited in its repair capacity and it is only when this constitutive system can not repair the damage rapidly enough, that the adaptive response mechanism is induced (Schendel *et al.* 1978). The nucleotide sequence of *ogt* is distinct from the *ada* gene however, the translated amino acid sequences of these two transferases reveal extensive regions of homology. (Takano, Nakamura and Sekiguchi 1991; Friedberg, Walker and Wolfram 1995).

The adaptive response in other organisms

An adaptive response against environmental alkylating agents appears to be widespread in microorganisms. Among prokaryotes, 33 species of Gram-negative bacteria belonging to 19 genera exhibit the adaptive response mechanism along with the enteric Gram-positive bacteria *Bacillus subtilis* and *Micrococcus leutus* (Friedberg, Walker and Wolfram 1995). Unlike *E. coli* the activity of the methyltransferase in *B. subtilis* is separated into two proteins a 24 kDA AdaA and a 20-kDa AdaB, both showing high sequence conservation with *E. coli* Ada and Ogt (Friedberg, Walker and Wolfram 1995). However, the *B. subtilis* Ada promoter does not show any sequence homology with the Ada box promoter region of the *ada* and *alkA* genes of *E. coli* (Sedgwick and Vaughan 1991). The two *ada* genes have distinct roles in the adaptive

response process, the *adaA* gene appears to be responsible for transcriptional activation of the *ada* operon, while the *adaB* gene encodes for the alkyltransferase (Friedberg, Walker and Wolfram 1995). Unlike *E. coli* or *B. subtilis*, *M. leutus* contains three distinct methyltransferases that repair O^6-methylguanine, O^4-methylthymine and methylphosphotriesters (Friedberg, Walker and Wolfram 1995). Despite the fact that *S. typhimurium* and *E. coli* share 75% amino acid sequence identity in their Ada protein, *S. typhimurium* exhibits only a weak induction of the adaptive response. The poor transcriptional activation of *ada* exhibited in *S. typhimurium* has been attributed to self methylation (Vaughan and Sedgwick 1991). Studies suggest that in this organism the Ogt protein is responsible for the majority of alkylation repair (Yamada *et al.* 1995). Although the adaptive response is found across a variety of microorganism, this inducible repair mechanism does not appear to be conserved in *Haemophilus influenzae, Staphylococcus aureus, Lactococcus lactis* or *Neisseria gonorrhoeae* (Friedberg, Walker and Wolfram 1995).

Methyltransferase activity has been documented in fish, *Drosophila melanogaster*, *Saccharomyces cervisia*, *Aspergillus nidulans*, and in mammalian cells (Friedberg, Walker and Wolfram 1995). There appears to be extensive conservation in the predicted amino acid sequence of O^6-alkylguanine DNA alkyltransferase genes across these organisms especially in the coding regions for the enzyme's active site. The functional properties of mammalian O^6MGT are similar to those of *E. coli* O^6MGT I in its substrate specificity and irreversible inactivation, and resembles *E.coli* O^6MGT II in its non-inducibility and inability to repair alkylphosphotriesters in DNA (Friedberg, Walker and Wolfram 1995). The adaptive response has been investigated in

transformed Chinese hamster ovarian cells and human skin fibroblasts. Continuous exposure to low doses of MNNG resulted in an increased resistance to further alkylation damage (Friedberg, Walker and Wolfram 1995). In humans the alkyltransferase gene is located on chromosome 10 and spans over 170kb. Endogenous cellular processes and chemotherapeutic nitrosoureas such as procarbazine and temozolamide have severe cytotoxic mutagenic and carcinogenic effects in humans (Christians and Loeb 1996; Sedgwick and Vaughan 1991). O^6-methylguanine has been implicated in playing a major role in the production of tumors by alkylation carcinogens (Friedberg, Walker and Wolfram 1995) and alkyltransferases seem to be effective in the treatment of human malignancies (Christians and Loeb 1996).

A variety of methylating agents can be found in the environment. Bacteria are often attracted to nutrient rich soils that contain significant levels of methylating agents. These agents can be secreted by bacteria such as the antibiotic Streptozotocin secreted by *Streptomyces achromogens.* This antibiotic is similar to MNNG in its ability to induce the adaptive response (Sedgwick and Vaughan 1991). Precursors common in decaying matter form compounds such as methylurea and methylnitroguanidine (Sedgwick and Vaughan 1991). Nitrosamides are strong candidates for adaptive response induction and are formed by the chemical or enzymatic nitrosation of amides (Vaughan *et al.* 1991). This synthesis can occur at pH levels often found in acid soils and polluted waters (Vaughan *et al.* 1991; Sedgwick and Vaughan 1991).

1.2.3 Research Objective

The research conducted in this laboratory aims at studying the adaptation of *E. coli* to increased levels of mutagens, specifically EMS. Previous studies have primarily observed *E. coli's* ability to acclimate phenotypically over very short time intervals to EMS. However, it is impossible to observe the effects of mutagenic agents on organisms and in turn on populations in such short term analysis, since new mutations may not necessarily contribute extensively to genetic variation relative to that which is already present (Bennett and Lenski 1993). Therefore the analysis discussed here is focused upon the adaptation of *E. coli* exposed to EMS over longer evolutionary time scales (Travisano *et al.* 1995) and can be separated into the following components: (i)General characterization of adapted *vs* the unadapted populations of *E. coli*, (ii) Adaptive response in adapted *E. coli* cell lines compared to unadapted lines, (iii) Fitness response of adapted *vs* unadapted populations of *E. coli* in the presence of alkylation treatment, (iv) Actual sequence changes involved in the adaptation of *E. coli* to mutagens.

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Chapter 2

General characterization of the adapted cell lines

2.1 Introduction

One of the key elements of biological evolution is the adaptation of organisms to their environment. Evolutionary biologists endeavour to elucidate the dynamics of these processes and to understand the contributions of natural selection, chance events and historical constrains (Lenski *et al.* 1991). A wide range of evolutionary questions have been addressed using long-term selection experiments with microbial populations (Elena and Lenski 1997; Korona 1996). It has been suggested that the large populations sizes of microbial organisms is the key factor in their ability to survive diverse and extreme environmental conditions (Koch 1993). Microbial organisms

are continually exposed to a variety of natural mutagens within their environment and therefore have evolved mechanisms to protect against the toxicity and mutagenic effects of these agents (Sedgwick and Vaughan 1991). A significant portion of naturally occurring mutagens and carcinogens consist of alkylating agents (Horsfall *et al.* 1990). An example of a powerful alkylating agent is methanosulfonic acid ethyl ester (EMS) which primarily produces potentially mutagenic O^6-alkylguanine lesions (Sledziewska-Gojska and Torzewska 1997; Horsfall *et al.* 1990) and to a lesser extent lethal 3-methyladenine and 3-methylguanine lesions (Friedberg, Walker and Wolfram 1995). O^6-alkylguanine lesions result in mispairing of a guanine with thymine during DNA synthesis, on the other hand 3-methyladenine and 3-methylguanine projects its methyl group into the minor groove and blocks this region of the DNA helix (Friedberg, Walker and Wolfram 1995). In *Escherichia coli* O^6-alkylguanine repair is mediated by two different of repair enzymes, Ogt, a constitutively expressed transferase (19-kDa) and the inducible transferase Ada (37-kDa) (Roldan-Arjona *et al.* 1994). Lesions caused by 3-methyladenine and 3-methylguanine are repaired by DNA glycosylases; *tag* a constitutive form and *alkA* an inducible form. The O^6-methyl transeferase I (O^6 MGT I) or Ada protein acts to reduce alkylation damage by transforming the methyl group from the alkylated DNA to its own cystein residue leaving behind an unmodified guanine base (Demple *et al.* 1982; Lindahl *et al.* 1988). The AlkA protein on the other hand does not reverse base damage but rather excises the 3-methyladenine and 3-methylguanine lesions directly from the DNA (Friedberg, Walker and Wolfram 1995; Karran, Hjelmgren and Lindahl 1982). Both of these mechanisms are components of a larger repair complex coined the Adaptive Response. Studies have indicated that upon continuous exposure of *E. coli* to low doses

of alkylating agents, *E.coli* cells develop significant resistance to the lethal and mutagenic effects of higher challenge doses of alkylating agents. This inducible Adaptive Response repair mechanism is distinct from UV-induced error-prone SOS repair, and allows increased survival at little cost to the cell and results in a significantly reduced mutation frequency compared to non-adapted cells (Lindahl *et al.* 1988; Samson and Cairns 1977). To date, "Adaptive Response" studies conducted in *E. coli* have primarily looked at the induction of the repair mechanism following phenotypic adaptation with low doses of alkylating agents for very short time intervals. It is almost impossible to observe the genetic effects of a mutagenic agent on an organism or on populations in such short term analysis, because new mutations may not necessarily contribute extensively to genetic variation relative to that which is already present (Bennett and Lenski 1993). In this analysis a long-term evolutionary experiment was conducted in order to investigate genetic adaptation of *E. coli* populations exposed to low doses of the alkylating agent EMS for over 4000 generations.

2.2 Materials and Methods

Bacterial Lines. The experiments were conducted using a cell line isolated from a single isolated colony of *E. coli* CSH100 *ara* Δ (*gpt-lac*)5 mating type F′ *lac pro* A^+, B^+ ($lacI^Q lacPL8$) clone obtained from Cold Spring Harbor Laboratories (Miller *et al.* 1977). This strain was separated into six individual lines that were then maintained by serial culture as pure lines for over 10 months. At this point in time these ancestral cell lines ("generation 0") were further propagated for an additional 24 months (over

4000 generations) for long-term adaptation experimental analysis.

Chemical Reagents. Methanesulfonic acid ethyl ester (EMS), agar and all necessary supplements were obtained from Sigma Chemical Company.

Bacterial Growth Medium. Liquid growth media for daily culturing consisted of minimal M9 solution supplemented with 20% glucose, 1M $MgSO_4$, and 0.5% thiamine per litre.

Culture Conditions. Three replicates of two experimental treatment lines were established and maintained with daily continuous culturing at 37^0C. All lines were propagated with M9 minimal medium and maintained daily by 1:100 serial dilution into 10 ml of fresh medium. The EMS adapted lines designated as "E" were treated daily with 25 μl of EMS following serial dilution. The control lines designated as "C" were grown in the absence of EMS. All other conditions were identical for both the "E" and the "C" lines. In most of the experiments conducted a third cell line was established using an inoculate from the "E" EMS adapted line. This line, designated "e", was grown up overnight (approximately 8 generations) in the absence of EMS in order to eliminate physiological changes and distinguish between phenotypic acclimation and genetic modification.

Controls. In order to control for cross contamination and monitor for external contamination between cell lines, cell lines were established carry alternating arabinose marker states. This marker state remained consistent for the control "C" cell lines throughout the 4000 generations of long-term culturing. However, the arabinose marker state did not appear stable in the presence of the potent mutagen EMS and

continuous reversions were observed in the adapted "E" culture. As a result other stringent precautions were taken to monitor contamination. Designated pipetting equipment and culturing locations were established for the two groups to prevent any contamination between adapted and control cell lines. In order to prevent external bacterial contamination, no other bacterial cells were cultured with the designated equipment or in the vicinity the culturing locations. All six cell lines were monitored daily for cell lysis or the presence of cellular debris in liquid culture. Weekly, all cell lines were streaked out and screened for colony morphology, colour and for the presence of papillae. All nutrient supplements and culture mediums were sterilized and monitored for bacterial contamination on the regular basis. If contamination was suspected at any point of the quality control testing, media, supplements and cultures would be discarded and the latest frozen culture samples would be used to resume culturing.

Antibiotic resistance assay. Frozen samples of the original CSH100 strain and "C1", "C2", "C3", "E1", "E2", and "E3" dated at 1 generation (September 13 1996), 1200 generations (April 11 1997) and 3400 generations (April22, 1998) relative to the start of long-term experiment were grown up overnight and streaked out onto LB agar plates. Single colonies were picked at random and used to inoculate six microtitre wells for each sample containing 200μl of LB media. Cultures were grown up overnight at 37^0C. Samples were replica plated onto varying concentrations of LB agar plates containing either nalidixic acid, rifampicin, tetracycline, ampicillin, streptomycin, or kanamycin. Plates were left overnight, at 37^0C and scored for antibiotic resistance or sensitivity.

LAC assay. Frozen samples of the original CSH100 strain, control strains: CSH100, DH5α, LE392 and long-term culture cell culture lines "C1", "C2", "C3", "E1", "E2", "E3" strains from generation 1 (September 13, 1996) and 3400 generations (April22, 1998) were grown up overnight and streaked out onto LB agar plates. Six single colonies were picked at random and used to inoculate six microtitre wells containing 200μl of LB media respectively. Cultures were grown up overnight at 37^{0}C. Samples were replica plated onto LB agar plates containing 50μg/ml Xgal glucose indicator plates. Plates were left overnight at 37^{0}C and colonies were scored for blue colouring an indication of the constitutive level of β-galactosidase.

Isolation of genomic DNA. 5ml cultures were grown up for 4 hours at 37^{0}C. Cells were pelleted and washed with 2.5ml TES [10mM Tris, 25mM EDTA, 150mM NaCl, pH 8.0], washed again with 2.5ml $T_{10}E_{25}$ [10mM Tris, 25 mM EDTA, pH 8.0] and incubated with 250μl of lysozyme for 15 min at 37^{0}C followed by the addition of 300 μl of sarkosyl-protease and an additional incubation for one hour at 37^{0}C. Lysates were extracted twice with phenol-chloroform and once with chloroform. The aqueous phase was precipitated with 150 μl of 5M ammonium acetate and 0.5 volume of isopropanol gently layered on top. The two phases were mixed by gentle tapping of the centrifuge tube. Precipitated DNA threads were removed with a sterile glass pasteur pipette and washed with 90% ethanol followed by 70% ethanol and resuspended in 100 μl of TE[10mM Tris, 1 mM EDTA, pH 8.0]. DNA extracts were stored at -20^{0}C.

Rep PCR analysis. DNA was extracted from frozen samples as described above. Repetitive DNA PCR sequencing was conducted on the CSH100 strain, long-term culture strains "C1", "C2", "C3", "E1", "E2", "E3" at generation 1 (September 13,

1996) and 3400 generations (April22, 1998) and control strains CSH100, CSH101, LE392, JM101. Repetitive sequence elements were amplified using rep 1R-1a/2-1 primer combination in one reaction and 1R-1 primer in another reaction (REP 1R-1a sequence: 5'-III ICG ICG ICA TCI GCC-3', REP 2-1 sequence: 5'-ICG ICT TAT CIG GCC TAC-3'). PCR was performed using 2μl of genomic DNA extract, 6μl of $MgCl_2$, 5μl of 10x reaction buffer, 1μl of a 400μmol solution of each dNTP, 5U of AmpliTaq Gold DNA polymerase (PERKIN ELMER) and 2μl of a 25pmol/μl primer solution. PCR cycling conditions were as follows:total 30 cycles, preheat 12 min at 95^0C,denaturation 45 sec at 94^0C, annealing 90 sec at 45^0C and synthesis for 150 sec at 72^0C with an additional extension phase for 300 sec at 72^0 following the final cycle. 5μl aliquots of PCR amplification products were separated by length in a 0.7% agarose gel stained with ethidium bromide.

Survival Assay. Three 100 ml LB cultures were generated using a 5ml inoculate from overnight "E","C" and "e" cultures. All three lines were grown to log phase in LB media (approximately 4h) at 37^0C."E" cultures were grown in the presence of 250μl of EMS. Each culture was diluted with appropriate amounts of fresh LB followed by the addition of a 200μl challenge dose of EMS in a 10ml culture. Samples were taken every 10 min for one hour and thereafter every hour for an additional three hours during exposure to challenge EMS. Samples were then appropriately diluted and plated on LB. Plates were scored for survivors after 18-24 hours at 37^0C.

Mutation Frequency Analysis. The bacterial lines were also examined for mutation frequency during timed exposure to a 200μl (/10ml media) EMS challenge. The experiment was conducted as described above with the addition overnight cul-

tures generated from the timed samples, in order to allow segregation and expression of mutant phenotypes (Miller 1992). Samples from the overnight cultures were plated on LB+streptomycin and LB+nalidixic acid to determine the frequency of $Strep^r$ and Nal^r mutants respectively. Mutants were scored after 18-24h at 37^0C.

Disk Assay. Cultures were spread over the surface of M9 glucose minimal plates in order to produce bacterial lawns followed by a 2 hour incubation at 37^0C. Three replicate lines of each culture group "C","E" and "e" were tested. Following incubation, concentrations of 100μl ddH_2O, 50μl EMS/50μl ddH_2O, 75μl EMS/25μl ddH_2O and 100μl of EMS were applied to 2.4cm filter disks (Whatman Grade 1) placed equal distances from each other on M9 glucose plates containing a lawn of bacteria. Following incubation for 48 hours at 37^0C bacterial growth would occur in a ring around the disk containing the mutagen. The distance from the perimeter of the disk to the edge of the halo or point of bacterial growth (zone of inhibition) was measured at five equidistant points. The above experiment was then repeated using MNNG (1mg/ml).

2.3 Results

Antibiotic assay. Antibiotic assays were conducted on founding, ancestral and evolved *E. coli* strains. All six randomly sampled CSH100 founding line colonies produced no significant growth on plates containing ampicillin, streptomycin, nalidixic acid, kanamycin or tetracycline both at low and high concentrations. However significant cell growth was observed by all six colonies on rifampicin plates even at high concentrations (150μg/ml) indicating a strong genetic resistance to rifampicin (Table

2.1). Antibiotic assays on the evolved cultures over the 3400 generations of culturing indicate a consistent sensitivity to the antibiotics streptomycin and kanamycin for both control "C" and adapted "E" cell lines (Table 2.2). This is also true for nalidixic acid and cultures "C2", "C3", "E2", and "E3". Cultures "C1" and "E1" show a slightly higher tolerance for nalidixic acid at generation 1 with 3/6 and 2/6 randomly selected colonies showing significant cell growth at high concentrations of nalidixic acid. However, by generation 3400 no visible cell growth is observed in either of the cell lines in the presence of nalidixic acid. Control "C" cell lines show a consistent resistance to the antibiotic rifampicin over the 3400 generation of culturing with significant cell growth observed for all sampled colonies. The "E" cell lines on the other hand appear relatively sensitive initially at generation 1 and appear to have developed complete resistance to the antibiotic with all colonies sampled having significant growth in the presence of high doses of rifampicin. This gradual development of antibiotic resistance/tolerance appeared to be highly prevalent for the antibiotics tetracycline and ampicillin. All three control cell lines appear to develop a gradual increase in their dosage tolerance with regards to tetracycline concentrations. This change is significantly more dramatic in the adapted "E" cell lines where the initial sample indicated high sensitivity at generation 1, and yet by generation 3400 all three cultures have developed complete resistance to very high doses of tetracycline. Ampicillin assays indicate this same phenomenon with cultures that were entirely or slightly resistant becoming completely resistance to very high doses of ampicillin by generation 3400.

LAC assay. β-galactosidase assays were conducted for the CSH100 founding

strain, the ancestral strains (generation 1) and the evolved strains (generation 3400) (Table 2.3). The CSH100 founding strain and ancestral strains produced colonies of pale blue colour on X-gal M9 glucose indicator plates, similar to the LE392 a weak positive control strain. The evolved strains produced colonies of intermediate blue colour. These were consistently darker than the ancestral strains and yet significantly lighter than the BL21 positive control strain. The founding CSH100 strain and ancestral strains could grow on M9 lactose media but no growth was observed for the evolved strains on M9 lactose media. This experiment was replicated using two new controls; K-12; a positive wild-type strain and MC4100 a strain with a *lac* deletion. These results are based upon visual examination of the plates after 12 hours. It should be noted that during the experiment the last two colonies for the MC4100 strain were contaminated by mispuncturing the well with a toothpick containing the K-12 bacterial strain resulting in the K-12 wildtype phenotype for the last two wells. The CSH100 founding strain produced white colonies on Xgal alone and colonies of an intermediate blue colour on Xgal with IPTG, and showed substantial growth on minimal lactose plates. Ancestral 1996 samples for "C1", "C2", "C3", "E1", "E2", and "E3" resulted in either white or very pale blue colonies on the Xgal indicator plates. All three "C" lines showed full growth on minimal lactose media and produced blue colonies on Xgal containing IPTG. The "E" adapted ancestral cell lines showed no growth or very sparse growth on the minimal lactose plates and the "E1" and E3 lines produced white colonies on Xgal plus IPTG while "E2" produced blue colonies. Evolved cell lines from the 1997 and 1998 samples for all three "C" cell lines as well as all three "E" cell lines produced colonies of a medium blue colour that was significantly lighter than the colour produced by the K-12 strain when tested on

Xgal indicator plates. On the minimal lactose plates no visible growth was observed for the "C" cell lines and "E" lines showed no or very sparse growth on the lactose plates. Overall it appears that the evolved cell lines have a darker colour on Xgal indicator plates indicating higher β-galactosidase levels while the same strains have lost or have reduced their ability to use lactose as a carbon source. The Xgal indicator plates containing IPTG suggest that the induced levels of β-galactosidase levels either remain unchanged over the 4000 generations or become reduced with all generation 4000 samples for all lines producing very light blue or white colonies.

REP PCR analysis. The profile descriptions given below reflect both bands observed and recorded . Some of the bands recorded manually were very faint and are therefore appear barely visible or not visible at all in the photograph. Further analysis was not conducted due to time constraints and because this analysis was to gain a general profile of band patterns and not to determine specific band sizes, number of bands, deletions or insertions. However, the PCR amplification was repeated and the amplification products were consistent with respect to a general banding pattern and varied only in band intensity. The REP 1R-1a/2-1 primers produced a very similar band pattern for the strains LE392, CSH101, JM101 and CSH100(B), sharing a band at approximately 500bp in length and one around 600bp in length (Figure 2.4(a)). The founding CSH100(B) and the ancestral "C1", "C2", "C3", "E1", "E2", and "E3" strains produced identical banding patterns sharing four identical bands approximately 400, 500, 600 and 1800bp in length. The profiles produced by the evolved strains are very different from their ancestral counterparts (Figure 2.4(b)). The evolved "C1", "C2" and "C3" strains share only three bands at approximately

400, 500 and 600bp in length, their ancestral counterparts. However, these three evolved strains produced identical banding patterns varying only in band intensity. The banding pattern for evolved "E1", "E2" and "E3" strains also differs significantly from their ancestral counterparts sharing only one band approximately 500bp in length. Again, the "E1", "E2" and "E3" evolved strains are identical in banding patterns and differ from the evolved control strains. The evolved and the control strains appear to share four bands approximately 300, 500, 1000 and 1400bp in length. Like the 1R-1a/2-1 primers, the 1R-1a primers produced very similar yet not identical patterns for strains LE392, CSH101, JM101, CSH100(H) and CSH100(B) (Figure 2.4(c)). These strains appear to have five common bands approximately 1000, 1300, 1400, 1500, 1600 and 1900bp in length. All of the bands except for the 1500bp are also shared by the ancestral "C2", "C3", "E1", "E2" and "E3" strains (Figure 2.4(d)). The three control ancestral strains are identical in banding pattern yet differ from the "E" lines slightly by two bands at approximately 1630 and 2300bp in length. The "E2" strain appears to contain two extra bands at 1500 and 3200bp in length when compared to its "E1" and "E3" counterparts. The evolved strains differ significantly from the ancestral strains for both the control and the adapted strains. Both the evolved "E" adapted strains and the evolved "C" control strains produced identical banding patterns. These strains appear to have only retained the ancestral bands approximately 500, 1000, 1300, and 1500bp in length and have lost the remaining bands found in the ancestral strain profiles.

Survival. Figures 2.2 (a), (b) and (c) are the survival curves following a 200μl challenge dose of EMS. Each point represents the average survival of three replicate

experiments following EMS challenge. For all three groups of cell lines (groups #1, #2 and #3), the control line "C" initially began with a significantly higher number of cells ranging from $1.12x10^7$ to $1.4x10^8$ cells/ml. This initial starting concentration was significantly lower for the "e" and the "E" groups ranging from $4.35x10^6$ to $4.8x10^7$, and $3.25x10^6$ to $3.4x10^7$ respectively. The "C" control group exhibits an immediate decline in survivorship upon exposure to the challenge dose of EMS and this rapid decline continues till complete cell death is reached between 2 and 3 hours following exposure to the challenge dose of EMS. This rapi decline in cell survival is not observed in either of the adapted "E"/"e" cell lines. These cell lines remained relatively constant in cell survival as exhibited in Figure 2.2(c) or exhibited a slight growth phase in the initial 120 minutes following challenging dose of EMS as seen in Figures 2.2(a) and (b). Survivorship for these cell lines begins to decline at around 120 minutes following EMS challenge and continues to decline slowly until complete cell death is reached between 4 and 12 hours. LT_{50} values were determined for each cell line by extrapolation from the corresponding survival curves (Table 2.4). This value is a measurement of the time it takes for the cell line to be reduced to one half of its starting cell density following exposure to the challenge dose of mutagen. The LT_{50} values are significantly higher for the adapted "E"/"e" cell lines relative to the control "C" cell lines for all three groups tested. These results support the overall trend in the survival curves and suggest that the adapted cell lines have a higher tolerance to the toxic effects of the challenge dose of EMS relative to the control cell lines.

Mutation frequency. In Figures 2.3 (a)-(f) mutation frequencies of the three

cell lines ("C", "e" and "E") are plotted against time following a 200μl challenge dose of EMS. Nalidixic acid and streptomycin mutation frequency estimates indicate that the "e" and "E" adapted cell lines have a higher tolerance against mutagenic damage relative to control "C" evolved cell lines following the large challenge dose of EMS. Figures 2.3 (a), (b) and (c) indicate that the "E" cell lines have a relatively constant mutation frequency response from 10-120 minutes of challenge followed by complete culture death. Although these responses show a very minimal increase initally at approximately 10 minutes of challenge, this increase is followed by a decrease at approximately 30 minutes of challenge and this pattern appears consistent for nalidixic estimates for all three "E" cell lines; "E1", "E2" and "E3". Although the mutation frequency response curve varies between the three replicate cell lines the "e" lines consistently appear to be slightly more sensitive to the EMS challenge relative to the "E" lines yet more resistant relative to the control cell lines (Figures 2.3 (a), (b) and (c)). Among the three cell lines tested the "C" control group appears to have an increased sensitivity relative to the other two adapted cell lines showing significant increases in mutation frequency at approximately 40 minutes of challenge followed by a steady and rapid increase. The streptomycin estimates show similar trends (Figures 2.3 (e) and (f)). Again the "E" cell lines appear to have the highest resistance to the challenge EMS. The response for these lines remains relatively constant until approximately 30-60 minutes of challenge at which point the mutation frequency appears to increase signifcantly. The "e" cell lines appear to have comparable mutation frequency response to that of the "E" cell lines. These two lines also appear to have an enhanced resistance relative to the control cell line showing increase in mutation frequency at 10-40 minutes of challenge. Although the mutation frequencies appear

to increase at approximately the same time for all three cell lines, the rate of mutation accumulation appears to occur at a significantly reduced rate in the adapted cell lines.

Disk Assay. Disk assays were conducted on the "C", "e" and "E" cell lines in order to compare the zones of inhibition between the three cell lines. The zone of inhibition is the clearing between the disk where there is a very high mutagen concentration to the area of bacterial growth where the mutagen concentrations are diluted enough to permit cell growth. Measurements were taken at five equidistant points around the perimeter with the exception of areas of moisture or mutagen runoff which causes distortions in the ring of bacterial growth surrounding the zone of inhibition. These values were not used in the calculations since they would lead to inaccurate distance measures of bacterial growth and in turn mutagen tolerance. The values for each plate for all three estimates of the same group sampled on the same day (ie. "C1", "C2" and "C3") were averaged and the relative mean distance between the adapted "e" and "E" cell lines and the control cell lines "C" were calculated and graphed (Figure 2.8 and 2.10). The calculations were done in this manner to account for day to day variation found in the measurements due to varying amounts of moisture on plates. Excessive moisture on the plate surface tended to cause inflated estimates of the inhibition zones due to mutagen runoff from the disk which causes the diffusion gradient of the mutagen to spread further along the plate away from the filter disk. Figures 2.7 (a), (b) and (c) are examples of the disk assay plate zones on inhibition observed for the "C" lines, "e" lines and "E" lines respectively. On average the zones of inhibition values for the control "C" cell lines were significantly

larger than either the "E" and the "e" adapted strains for all concentrations tested suggesting an increased tolerance to higher EMS concentrations. The mean relative distances for each of the three concentrations tested were calculated for both the adapted "E" and "e" cell lines relative to the control "C" cell lines (Figure 2.8). These values indicate that the mean relative distance is significantly smaller for the two adapted cell lines. Between the two adapted cell lines, the relative distance measurements suggest that the "E" cell lines that are both genetically adapted and phenotypically acclimatized to the EMS appear to have a slightly higher tolerance to the mutagen compared to the "e" lines. Replicate experiments were conducted using the potent alkylating agent MNNG. All calculations were conducted as previously described with the EMS disk assays. The results of the MNNG assay also suggest a higher tolerance for MNNG in the adapted "e" and "E" cell lines relative to the control "C" cell lines (Figures 2.9 (a),(b) and (c) and 1.10).

2.4 Discussion

It is quite evident through the analysis conducted in this study that many genetic changes had taken place over the course of 4000 generations of bacterial propagation. These changes have affected antibiotic resistance, β-galactosidase levels, REP element fingerprints and lethal and mutagenic tolerance to large doses of alkylating agents.

The antibiotic assay results indicate that the bacterial strains used in our experiment were initially sensitive to all five of the antibiotics tested: streptomycin, ampicillin, tetracycline, nalidixic acid and kanamycin. This sensitivity remained rel-

atively constant for nalidixic acid, kanamycin and streptomycin. Any slight growth observed may be due to spontaneous mutants or slight variability of antibiotic concentrations on a plate. The gradual development of complete antibiotic resistance for the "E" adapted lines to rifampicin, and for both the "E" adapted and "C" adapted lines to ampicillin and tetracycline were quite unexpected. Antibiotic resistance is clearly beneficial to organisms exposed to antibiotics. However, one would expect that long-term culturing in an antibiotic-free environment would favour the selection of sensitive phenotypes.

There are a few examples in which there is an absence of cost associated with antibiotic resistance. Bacteria cells utilize inducible resistance functions in order to avoid cost of resistance in an antibiotic-free environment (Nguyen *et al.* 1989). However, in the absence of antibiotics resistant phenotypes are often associated with reduced fitness due to interference with normal physiological functions of the cell (Lenski 1997). Recent studies indicate that although resistant mutations may initially prove to be a burden to the cell, the actual fitness costs associated with the resistance may itself be subject to evolutionary change (Lenski 1997). Additional mutations are shown to occur that compensate for the physiological disruptions and thereby restoring normal cellular function to the cell without affecting the acquired antibiotic resistance (Schrag and Perrot 1996; Lenski 1997). Long-term serial culturing experiments indicate that mutations in the *rpsL* gene conferring streptomycin resistance to bacteria results in cells that have an increased fitness when competed in the absecnce of streptomycin, in comparison to their sensitive genetic counterparts. These mutations in the *rpsL* gene initially result in a significant decrease in peptide elongation. However,

over time this defect is eliminated with additional compensatory mutations that improve peptide elongation to a level comparable to the wildtype state without affecting streptomycin resistance (Schrag and Perrot 1996; Schrag, Perrot and Levin 1997). In *E.coli* K-12, spontaneous mutants possessing Tn-5 encoded bleomycin resistance have a significant fitness advantage over sensitive individuals during prolonged starvation. Unlike most other studies, this mutation provides an immediate advantage to the bacteria. The stability of the Tn-5 transposon encoded resistance is suggested to be due to enhanced repair of damage occurring during bacterial senescence (Blot, Meyer and Arber 1991).

The significant increase in resistance to ampicillin and tetracycline would not be as surprising in hindsight if this phenomenon were only found in the "E" adapted lines which were exposed continuously to EMS. In that case an increase in resistance may be accompanying the adaptations required for EMS resistance. However, the resistance was observed experiment wide in all three control and adapted cell lines suggesting that this phenomenon is not a result of the alkylation damage. The increased antibiotic resistance may in fact be a correlated response to adaptation to the stressful environment imposed daily on all six of the cell lines through daily serial culturing in a glucose limited environment. Recent studies indicate that many types of environmental stress imposed on microbial organisms result in increased antibiotic resistance both in the presence and in the absence of antibiotics. In most instances, *E. coli* do not usually respond to unfavourable environmental conditions through morphological changes, but rather undergo important changes in gene expression (Salah-Bey, Blanc and Thompson 1995). *ptr*, a multi-drug resistant gene found in *E. coli* has been shown

to become transcriptionally activated in response to stress associated with decreases in growth rate. *ptr* is believed to be part of a widespread stress-induced adaptive regulon that results in a 40 fold increase in transcription upon approach of stationary growth phase. *ptr* is just one of the various stress-induced families of genes which include the SOS regulon and the heat-shock regulon both of which are stress induced and provide resistance to a variety of antibiotics (Salah-Bey, Blanc and Thompson 1995).

Elevated humidity and temperature associated with hyperbaric conditions has shown to result in significant increases in bacterial resistance to a range of antibiotics (gentamicin, rifampicin and penicillin) (Hind and Attwell 1996). The resulting increase in antibiotic resistance was determined to be result of physiological changes induced in the bacteria as a result of the hyperbaric stress and not due to a reduction in antibiotic activity (Hind and Attwell 1996). Other studies have indicated that chronic metal stress affects microbial communities adversely with regards to bacterial diversity, biomass and activity and yet results in bacterial isolates highly tolerant to lead even without previous exposure to this substance. These lead resistance isolates were also found to have a unique antibiotic resistance profile that correlated with habitat (Roane and Kellogg 1996). These examples of evolutionary development of antibiotic resistance in bacteria suggests that the observations found in the antibiotic assay may not be surprising after all. Since the cultures were never exposed to antibiotics at any time point in their evolution aside from the testing, the appearance of the antibiotic resistance may be a consequence of hitchhiking with another adaptation and then maintained by the addition of compensating mutations.

It has been suggested that the compensating mutations themselves develop a genetic background in which sensitive revertants have a strong selective disadvantage, and an "adaptive valley" is produced separating the adaptive peaks of drug sensitivity and fitness compensated resistance (Schrag, Perrot and Levin 1997). That is, an evolved resistant strain would require at least two mutations to become a high fitness sensitive strain and individually each of these mutations would reduce the fitness of the strain (Schrag, Perrot and Levin 1997).

The CSH100 *E. coli* cell line (*ara* Δ (*gpt-lac*)5 mating type F′ *lacpro* A^+, B^+ (*lacI*^{Q} *lacPL8*)) contains a F′ plasmid carrying two mutations in the *lac* operon; a L8 CAP binding site which results in a 15 fold lowering of the maximum levels of Lac enzyme expression, and a I^Q mutation in the promoter that allows a 10 fold overproduction of the *lac* repressor. The light blue colour of the colonies formed by the CSH100 founding strain and ancestral strains are expected due to the genetic $I^+P^-Z^+$ properties of the CSH100 cell line. The P^- property of this strain does not lower the expression of Lac enzymes sufficiently to prevent growth on lactose minimal media and therefore the slight growth of these strains on the lactose minimal plates is not surprising (Miller 1992). Changes were observed in the evolved strains with regard to Lac enzyme expression. Evolved strains produced colonies with a darker blue colour than the ancestral strains, yet it was significantly lighter than the positive control BL21 or K-12 cell lines. However, no growth was observed on lactose minimal plates suggesting a decrease in the ability of the cultures to utilize lactose as a carbon source.

Overall this analysis suggests an slight increase in level of constitutively expressed

lac enzymes but decreased ability to utilize lactose as a carbon source and reduced induction levels of β-galactosidase. The reduced ability of the cell lines to utilize lactose as a carbon source may simply be due to the lack of lactose used in the culture media over the long-term evolution experiment and as a result glucose uptake efficiency was most likely enhanced while lactose uptake was not needed and therefore the ability to utilize lactose in these cells lines could be greatly reduced. The darker blue colouring of the colonies suggest increased β-galactosidase levels in the evolved strains in both control and EMS adapted lines may be a consequence of increased membrane permeability of Xgal into the cells. However, there is insufficient evidence to conclude this from our results. Further analysis is required to quantitate β-galactosidase levels. An ONPG β-galactosidase assay would enable quantification of β-galactosidase levels and determine if the observed conflicting results and merely due to enhanced membrane permeability and in turn greater cell uptake of Xgal.

The PCR primers used to conduct the REP analysis are complementary to conserved Repetitive Extragenic Palindromic (REP) sequences in the *E. coli* genome. There are 581 copies of REP sequences distributed throughout the *E. coli* chromosome, enabling random amplification of genomic segments (Nakatsu *et al.* 1998). Numerous roles have been proposed for these elements such as transcriptional termination, mRNA stability and chromosomal domain organization (Versalovic, Koeuth and Lupski 1991). These extragenic repetitive sequences have recently been used to fingerprint bacterial genomes. Prior to REP-PCR, t-DNA PCR fingerprint analysis was commonly used. This technique uses repeated t-RNA genes as consensus primer binding sites to directly amplify DNA fragments of different sizes, and unlike

REP-PCR, t-DNA PCR is invariant between bacterial strains of a given species and between related bacterial species (Versalovic, Koeuth and Lupski 1991). One of the features of the REP primers used in this analysis is the use of inosine at several sites in order to increase the number of hybridization sites. Inosine containes hypoxanthine a purine base which is capable of forming Watson-Crick base pairs with A, G, C and T (Versalovic, Koeuth and Lupski 1991). This analysis was done in order to obtain a general idea of the DNA profile for the ancestral strains and to determine if any changes in the DNA profile have occurred to the strains after 3400 generations of culturing in glucose limited cultures (with or without EMS treatment). The banding patterns produced by the Rep 1R-1a and 1R-1a/2-1 primers were similar yet not identical for the the strains LE392, JM101, CSH101 and CSH100 strains. Although these are closely related strains of the same bacterial species the pattern appears unique and demonstrates the high discriminating power of Rep PCR profiling between even closely related strains of the same species. The founding strain CSH100(B) and the ancestral strains produced identical patterns for primer 1R-1a/2-1 (Figure 2.4 (a)), and similar yet not identical patterns for primer 1R-1a(Figure (b)). There are two possibilities for these observations. With the low resolution in banding pattern due to varying intensities of the bands and due to electrophoresis conditions, it is quite possible that the two bands missing (500 and 900) in the ancestral patterns but present in the founding CSH100(B) are present but barely visible due to masking by surrounding bands of high intensity. A second possibility would be caused by running the ancestral strain samples on the lower portion of a double gel at which point the movement of ethidium bromide within the gel may have masked the faint these lower bands. The ancestral "E" strains were also found to differ from the founding strain

in banding pattern and the "E1" and "E2" differ significantly from the "E2" strains in pattern (Figure 2.4 (c) and (d)). These differences cannot be explained by band intensity or staining since the three ancestral samples produce different prints. It is quite possible that the pattern differences are a result of significant genetic changes in the species over the 10 months of culturing between the founding and ancestral lines in the presence of the EMS. It has been shown that large portion of the genetic adaptation occurs when organisms are initially exposed to environmentally challenging conditions (Lenski and Travisano 1994). The banding profiles for the evolved strains is significantly different from the ancestral strains for both the 1R-1a and 1R1a/2-1 primers, suggesting genomic changes in the cell lines over the 3400 generations of serial culturing. Restriction fragment length polymorphisms (RFLP) analysis using insertion sequence elements as probes have shown that evolving populations of *E. coli* become increasingly different from their ancestor over time with significant diversity accumulating in each population over 10,000 generations of serial propagation (Papadopoulos *et al.* 1999).

What is extremely interesting is the fact that there appears to be parallel evolutionary changes among the laboratory evolved replicate populations. The REP1R-1a/2-1 results show identical banding patterns for all three "C" control strains and identical banding patterns for all three "E" adapted strains, yet they differ significantly between the control and adapted strains sharing only 4 bands. In both cases the evolved strains have numerous additional bands absent in their ancestral counterparts. The 2-1 results indicate a similar parallelism among the strains, however in this case all the evolved lines (both "C" and "E") have developed identical banding

pattern and again these patterns differ significantly from their ancestral counterparts. This analysis suggests that significant genetic changes in REP patterns have occurred between ancestral strains and evolved strains over 3400 generations. These evolutionary changes were remarkably identical in REP PCR products between control and adapted cell lines when using the REP 1R-1a primers and within lines when using REP1a and REP 1R-1a primers. These observations are most likely the result of genomic rearrangements that may have occurred during 3400 generations of serial culturing.

The evolutionary parallelism observed in the cell lines between replicate populations in the antibiotic assay, β-galactosidae assay and with the REP PCR analysis is not unusual in long-term bacterial cultures (Korona 1996; Lenski *et al.* 1991; Lenski and Travisano 1994). Adaptations to a particular environment tend to be similar among replicate populations, yet the underlying genetics of the adaptation may be quite distinct (Hall 1988; Lenski 1988). Many different genetic changes can increase an organism's fitness in a particular environment, the similarity or the differences in these genetic changes is a result of both the number of different possible adaptive genetic changes and the frequency at which they occur (Nakatsu *et al.* 1998). REP PCR analysis in long term evolved populations of *Ralstonia sp.* indicated common genetic changes with regards to REP PCR products arising from genetic recombination mechanisms causing duplications and deletions of large spans of DNA sequences. (Nakatsu *et al.* 1998). Recent studies have indicated that recombination and genetic rearrangements may play a highly significant role in adaptive evolution (Nakatsu *et al.* 1998; Guttman and Dykhuizen 1994; Papadopoulos *et al.* 1999). It has been

suggested that this type of genetic re-organization is modulated by biochemical complexes that respond to physiological inputs and as a result promote processes such as gene shuffling, duplication and dispersal of gene sequences and amplification of repetitive DNA (Lenski and Sniegowski 1995). Our results indicate common genetic changes within the control cell lines and within the adapted cell lines and therefore suggests that these changes are environment specific.

The mutation frequency and survival assays conducted in this investigation strongly suggest that prior low dose exposure to EMS during long-term culturing results in a significant adaptation to larger challenge doses of EMS. The survival curves indicate a rapid decline in cell survival in the unadapted control "C" cell lines, immediately following initial exposure to the challenge dose of EMS and continues until complete culture death is reached between two and three hours. This is not observed in either of the adapted cell lines "E" or "e". These lines appear to resist the cell killing for a significantly longer period of time, demonstrating a slight initial growth phase followed by a slow decline that begins approximately 2h hours following initial exposure to the challenge dose of EMS and total culture death is reached on average at 4-18 hours following challenge dose of EMS. These results further support previous studies whereby upon continuous exposure of *E. coli* to low doses of the alkylating agent MNNG, *E. coli* cells develop significant resistance to the lethal effects of higher challenge doses of the mutagens (Samson and Cairns 1977; Vaughan *et al.* 1991; Schendel *et al.* 1978). However, these previous studies have looked at *E. coli's* enhanced cell survival through short term exposure to low doses of alkylating agents and are therefore looking at phenotypic acclimation resulting in induction of the adaptive

response repair pathway. In this study both the adapted cell lines "E" and "e" result in similar responses to the challenge dose of EMS. Since the "e" cell line is propagated overnight without the presence of EMS any physiological acclimation to the mutagen is eliminated and the results shown in the survival curves are due to long-term genetic adaptation. These cells lines show a similar response when exposed to a challenge dose of MNNG another powerful alkylating agent as discussed in Section 3 and Figure 3.3. This suggests a cross adaptation to MNNG and perhaps other alkylating agents.

The disk assay experiment is a rapid and effective semi-quantitative method of analysis often used to screen many different compounds for mutagenic activity and to observe the response of specific strains to the mutagens (Miller 1992). The results of the disk assay strongly support the survival curves and indicate an enhanced tolerance in the adapted cell lines to the challenge EMS dose relative to the control cell lines. These results also confirm cross adaptive tolerance to the mutagen MNNG and a slightly higher level of resistance in the "E" adapted cell lines compared to the "e" adapted lines.

Overall both nalidixic acid and streptomycin mutation frequency estimates suggest that both "e" and "E" cell lines have a higher resistance to the mutagenic effects of the EMS challenge. These lines appear to resist mutation induction for a period of time and begin to show a delayed or reduced mutation induction response. The non-adapted "C" lines do not appear to have this delayed induction and show increased sensitivity to the challenge EMS with regards to mutagenic damage. Overall the "E" adapted cell lines appear to have a higher level of resistance compared to the "e" adapted cell line. This may be due to the fact that the "E" cell line has developed

genetic adaptation and phenotypic acclimatization through continuous EMS exposure up until the time of the experimental EMS challenge resulting in an enhanced mutagenic resistance. The "e" cell lines on the other hand have genetic adaptation with continuous EMS exposure followed by an overnight interruption in exposure to the mutagen therefore removing the physiological adaptation from this cell line. The enhanced mutagenic resistance observed in the adapted cell lines supports previous studies showing significantly less his^+ and trp^+ reversions in the adapted cells following challenge doses of the potent alkylating agent MNNG suggesting enhanced resistance to both the lethal and mutagenic effects of large alkylating doses (Jeggo *et al.* 1977; Samson and Cairns 1977). However, unlike the previous studies conducted, these results indicate that long-term genetic adaptation to EMS can also result in an increased resistance to mutagenic damage relative to unadapted cell lines. The "e" and "E" cell lines commonly show a slow mutation phase followed by an enhanced mutation induction phase sometimes reaching levels observed in non-adapted cells. It has been suggested that the rapid delayed increase in mutation frequency seen in the adapted cell lines may be a consequence of the induction of error-prone repair which at this point would result in disguising any remaining adaptive effects (Schendel *et al.* 1978). MNNG mutagenesis studies suggest limited capabilities of the error-free repair pathways which can provide protection from low to moderate doses of alkylating agents (Lindahl *et al.* 1988; Schendel *et al.* 1978). Once significant amounts of lesions have accumulated, adaptive enzymes are induced in order to arrest any further damage. Studies suggest that with extended exposures or extremely high doses of alkylating agents the error-prone repair pathway is induced resulting in a rapid increase in mutation frequency as seen in these mutation response curves to EMS.

Mutagenesis studies indicate the inability of EMS to induce SOS functions (Jeggo *et al.* 1977; Lindahl *et al.* 1988), therefore if error-prone repair is the cause of the rapid increase then it must be due to some other type of pathway besides SOS. The distinct error-prone repair pathway previously attributed to the SOS repair pathway would be able to induce such an effect (Wang *et al.* 1995). This pathway termed UVM for UV modulation is strongly induced by alkylating agents and is found to be distinct from both the adaptive response and the SOS responses requiring neither the *ada, alkA, alkB, aidB or ogt* genes nor the *E. coli recA* gene.

Table 2.1: Antibiotic assay on the CSH100 bacterial strain. Six replicate microtitre overnight cultures of CSH100 were replica plated and scored for growth on LB agar plates containing various concentrations of antibiotics. (where Conc. is the concentration of each antibiotic in μg/ml and N is the count of resistant colonies).

Nalidixic acid		Rifampicin		Tetracycline		Ampicillin		Streptomycin		Kanamycin	
Conc	**N**	**Conc**	**N**	**Conc**	**N**	**Conc**	**N**	**Conc**	**N**	**Conc**	**N**
5	0	50	6	1	6	50	0	50	6	1	6
10	0	60	6	5	6	60	0	60	0	10	0
15	0	70	6	10	0	70	0	70	0	20	0
20	0	80	6	15	0	80	0	80	0	30	0
25	0	90	6	20	0	90	0	90	0	40	0
30	0	100	6	25	0	100	0	100	0	50	0
35	0	110	6	30	0	110	0	110	0	60	0
40	0	120	6	35	0	120	0	120	0	70	0
45	0	130	6	40	0	130	0	130	0	80	0
50	0	140	6	45	0	140	0	140	0	90	0
55	0	150	6	50	0	150	0	150	0	100	0

Table 2.2: Antibiotic assays on bacterial strains. Six replicate microtitre overnight cultures of "C1", "C2", "C3", "E1", "E2", and "E3" cultures were replica plated and scored for growth on LB agar plates containing various concentrations of antibiotics. Cell line samples were obtained from samples frozen down at various time points realtive to the start of the long-term experiment: G1(1 generation), G2 (1200 generations) and G3 (3400 generations)

Nalidixic acid

	C1			C2			C3			E1			E2			E3		
Conc	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3
5	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
10	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
15	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
20	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
25	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
30	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
35	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
40	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
45	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
50	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0
55	3	0	0	0	1	0	0	1	0	2	2	0	0	0	0	0	0	0

Rifampicin																		
	C1			C2			C3			E1			E2			E3		
Conc	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3
50	3	5	6	5	6	6	6	6	6	3	6	6	0	0	6	0	0	6
60	3	6	6	6	6	6	6	6	6	3	6	6	0	0	6	0	0	6
70	3	5	6	5	6	6	6	6	6	3	6	6	0	0	6	0	0	6
80	3	4	6	5	6	6	6	6	6	2	6	6	0	0	6	0	0	6
90	3	5	6	5	6	6	6	6	6	2	6	6	0	0	6	0	0	6
100	3	5	6	5	6	6	1	6	6	2	6	6	0	0	6	0	0	6
110	3	5	6	3	6	6	6	6	6	2	6	6	0	0	6	0	0	6
120	3	5	6	5	6	6	6	6	6	2	6	6	0	0	6	0	0	6
130	3	5	6	5	6	6	6	6	6	2	6	6	0	0	6	0	0	6
140	3	5	6	5	6	6	6	6	6	2	6	6	0	0	6	0	0	6
150	3	5	6	5	6	6	6	6	6	2	6	6	0	0	6	0	0	6

Tetracycline

	C1			C2			C3			E1			E2			E3		
Conc	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3
1	5	6	6	6	4	6	6	6	6	4	2	6	2	0	6	2	4	6
5	3	6	6	5	3	6	6	6	6	4	2	6	2	0	6	0	0	6
10	0	6	6	0	3	6	0	6	6	0	2	6	2	0	6	0	0	6
15	0	6	6	0	2	1	0	6	3	0	1	6	0	0	6	0	0	6
20	0	5	6	0	2	1	0	0	0	0	0	6	0	0	6	0	0	6
25	0	5	6	0	0	1	0	0	0	0	0	6	0	0	6	0	0	6
30	0	5	6	0	0	0	0	0	0	0	0	6	0	0	5	0	0	6
35	0	5	6	0	0	0	0	0	0	0	0	6	0	0	5	0	0	6
40	0	5	6	0	0	0	0	0	0	0	0	6	0	0	5	0	0	6
45	0	5	6	0	0	0	0	0	0	0	0	6	0	0	0	0	0	5
50	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Ampicillin

	C1			C2			C3			E1			E2			E3		
Conc	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3
50	3	6	6	0	2	6	0	6	6	2	2	6	2	2	6	0	0	6
60	3	6	6	0	2	6	0	6	6	2	2	6	2	2	6	0	0	6
70	3	6	6	0	2	6	0	6	6	2	2	6	2	2	6	0	0	6
80	3	6	6	0	2	6	0	6	6	2	2	6	2	2	6	0	0	6
90	3	6	6	0	2	6	0	6	6	2	2	6	2	1	6	0	0	6
100	3	6	6	0	2	6	0	6	6	2	2	6	2	2	6	0	0	6
110	3	6	6	0	2	6	0	6	6	2	2	6	2	2	6	0	0	6
120	3	6	6	0	2	6	0	6	6	0	2	6	2	2	6	0	0	6
130	3	6	6	0	2	6	0	6	6	0	2	6	2	2	6	0	0	6
140	3	6	6	0	2	6	0	6	6	0	6	0	2	2	6	0	0	6
150	3	6	5	0	2	5	0	6	5	0	6	0	2	2	6	0	0	6

Streptomycin																		
	C1			C2			C3			E1			E2			E3		
Conc	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3
50	0	6	0	0	0	0	0	6	0	0	0	6	0	0	5	0	0	3
60	0	6	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0
70	0	6	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0
80	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
90	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
110	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Kanamycin																		
	C1			C2			C3			E1			E2			E3		
Conc	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3	G_1	G_2	G_3
1	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2.3: Assay for colony colour on Xgal indicator plates.

Strain	**Colony colour on Xgal**
DH5α (negative control)	white
BL21 (positive control)	dark blue
LE392	pale blue
CSH100	pale blue
C1 1 Generation	pale blue
C2 1 Generation	pale blue
C3 1 Generation	pale blue
E1 1 Generation	pale blue
E2 1 Generation	pale blue
E3 1 Generation	pale blue
C1 3400 Generations	medium blue
C2 3400 Generations	medium blue
C3 3400 Generations	medium blue
E1 3400 Generations	medium blue
E2 3400 Generations	medium blue
E3 3400 Generations	medium blue

Figure 2.1: Assay for colony colour on Xgal indicator plates. 200μl microtiter wells were inoculated with K-12, MC4100, CSH100 founding strains, "C1" and "C2" cell lines from frozen samples dated ancestral (Sept 1996), generation 1200 (Sept 1997) and generation 4000 (Sept 1998) and replica plated on minimal lactose plates, X-gal and X-gal /IPTG plates. Row 1: K-12, row 2: MC4100, row 3: CSH100 founding strain, row 4: "C1" 1996, row 5: "C1" 1997, row 6: "C1" 1998, row 7: "C2" 1996, row 8: "C2" 1997

TOPA
lacZ
TOPA
lacY+A
TOP A
M9

Figure 2.2: Assay for colony colour on Xgal indicator plates. 200μl microtiter wells were inoculated with K-12, MC4100, CSH100 founding strains, "C2", "C3", "E1" and "E2" cell lines from frozen samples dated ancestral (Sept 1996), generation 1200 (Sept 1997) and generation 4000 (Sept 1998) and replica plated on minimal lactose plates, X-gal and X-gal /IPTG plates. Row 1: "C2" 1998, row 2: C3 1996, row 3: "C3" 1997, row 4: "C3" 1998, row 5: "E1" 1996, row 6: "E1" 1997, row 7: "E1" 1998, row 8: "E2" 1996

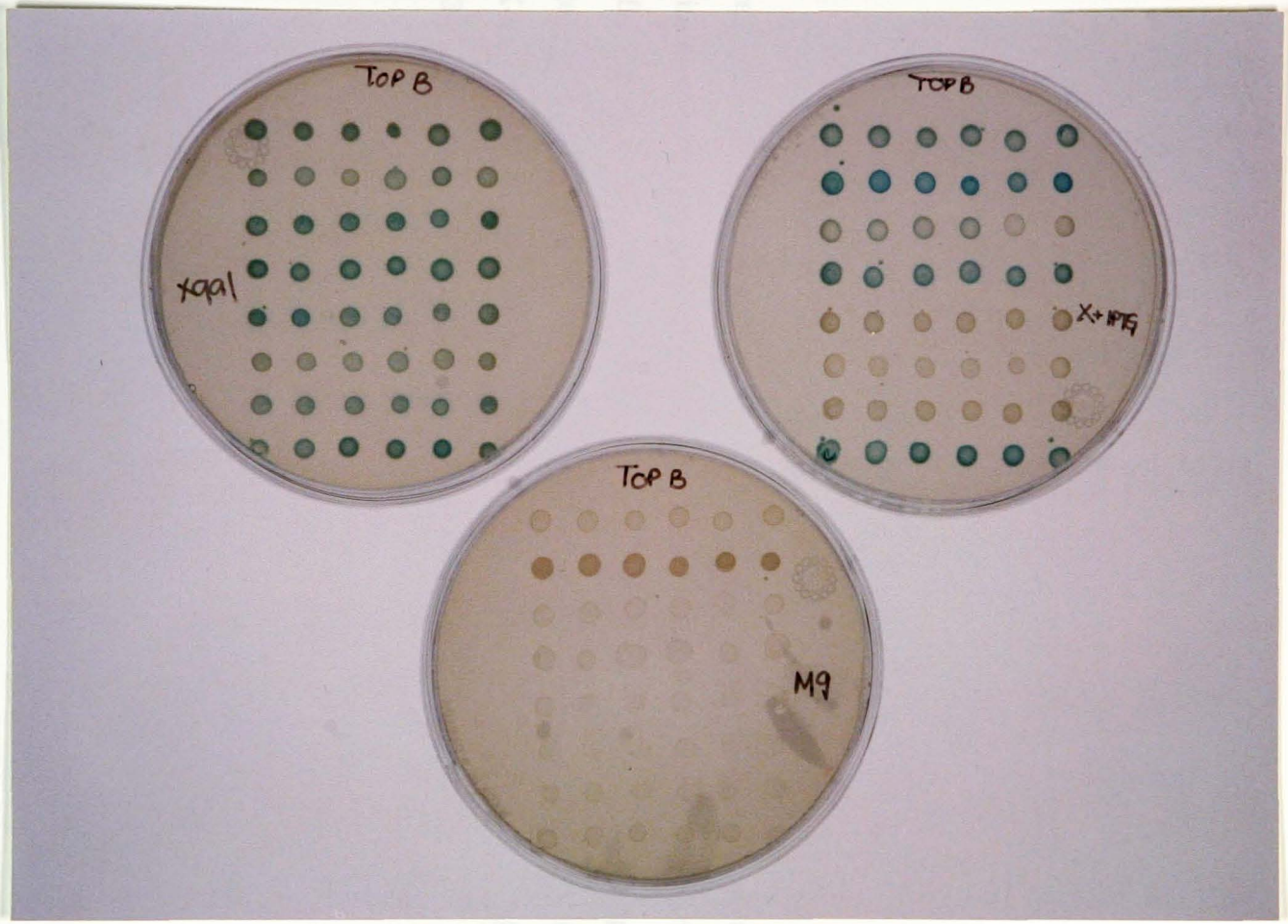

TOP B
xgal
TOP B
X+IPTG
TOP B
M9

Figure 2.3: Assay for colony colour on Xgal indicator plates. 200μl microtiter wells were inoculated with K-12, MC4100, CSH100 founding strains, "E2" and "E3" cell lines from frozen samples dated ancestral (Sept 1996), generation 1200 (Sept 1997) and generation 4000 (Sept 1998) and replica plated on minimal lactose plates, X-gal and X-gal /IPTG plates. Row 1: "E2" 1997, row 2: E2 1998, row 3: "E3" 1996, row 4: "E3" 1997, row 5: "E3" 1998, row 6: Negative Control

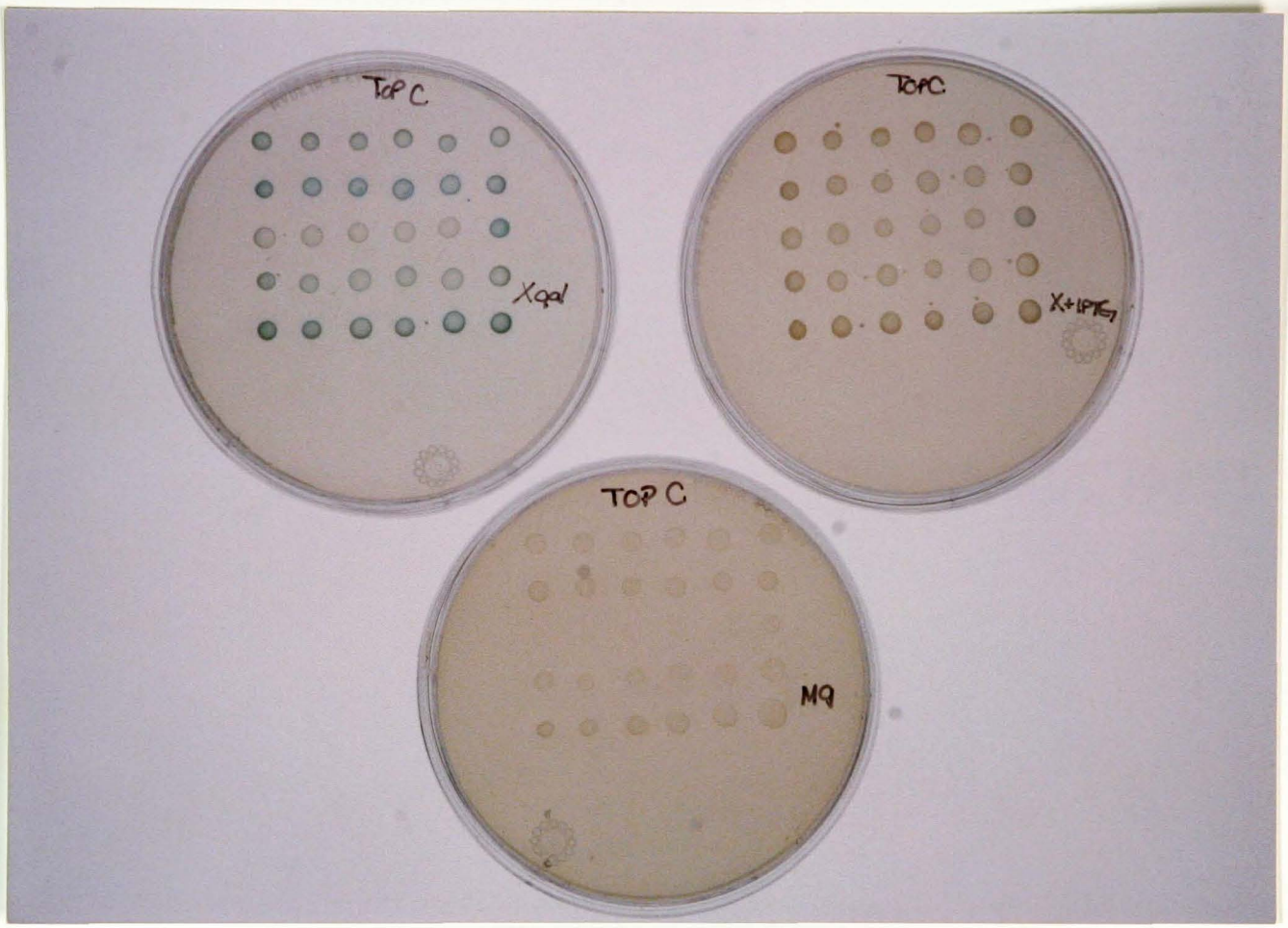
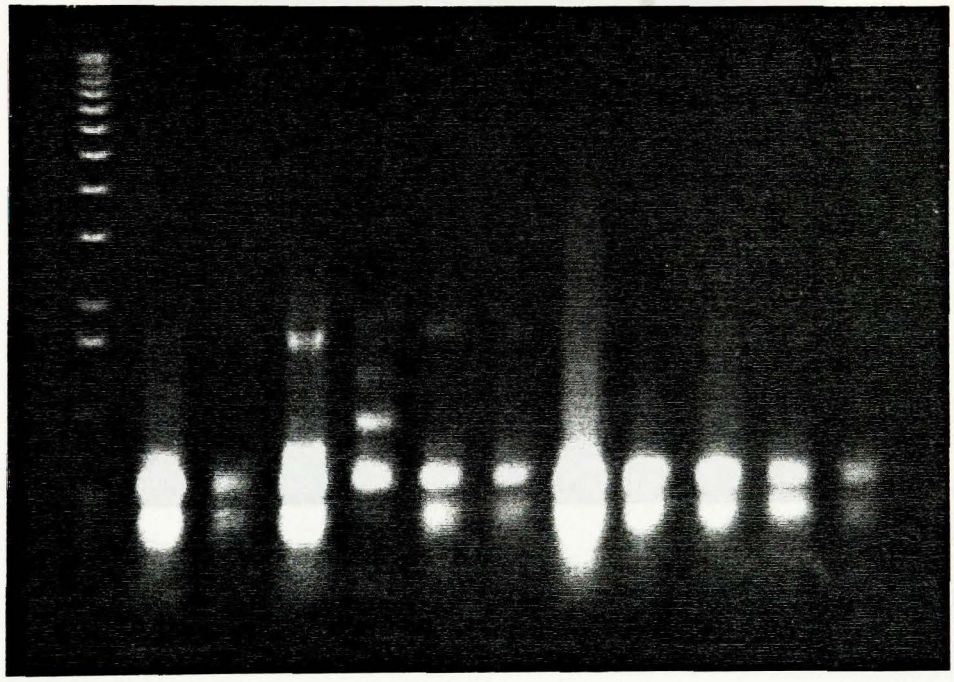
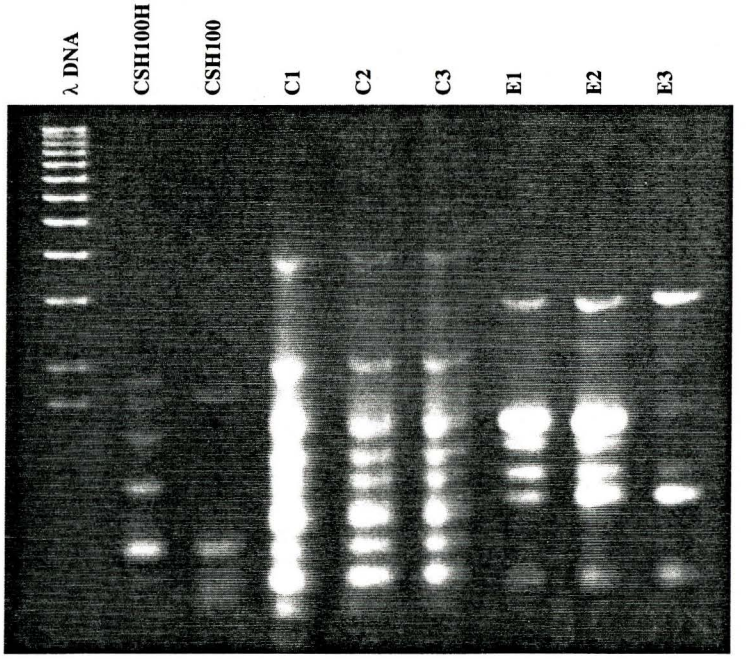

TOP C
Xgal
TOPC
X+IPTG
TOP C
MG

Figure 2.4: Repetitive DNA sequence element PCR (REP) analysis of bacterial strains using REP primers. Figures (a) and (b) primer IR-1a/2-1, Figures (c) and (d) primer IR-1. "C1", "C2", "C3", 'E1", "E2" and "E3" cell line samples were obtained at generation 3400

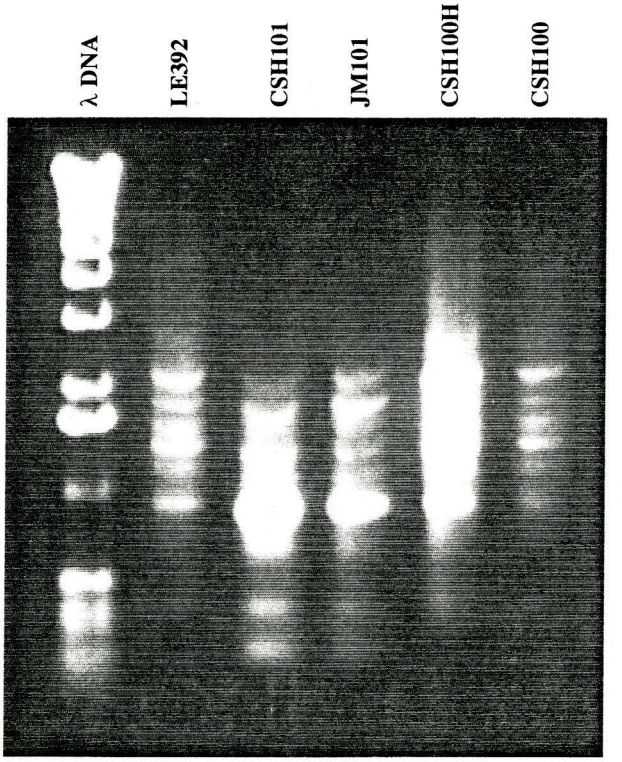
a


λ DNA
LE392
JM101
CSH101
CSH100H
CSH100
C1 ANCESTRAL
C2 ANCESTRAL
C3 ANCESTRAL
E1 ANCESTRAL
E2 ANCESTRAL
E3 ANCESTRAL
NEGATIVE

b



C



d

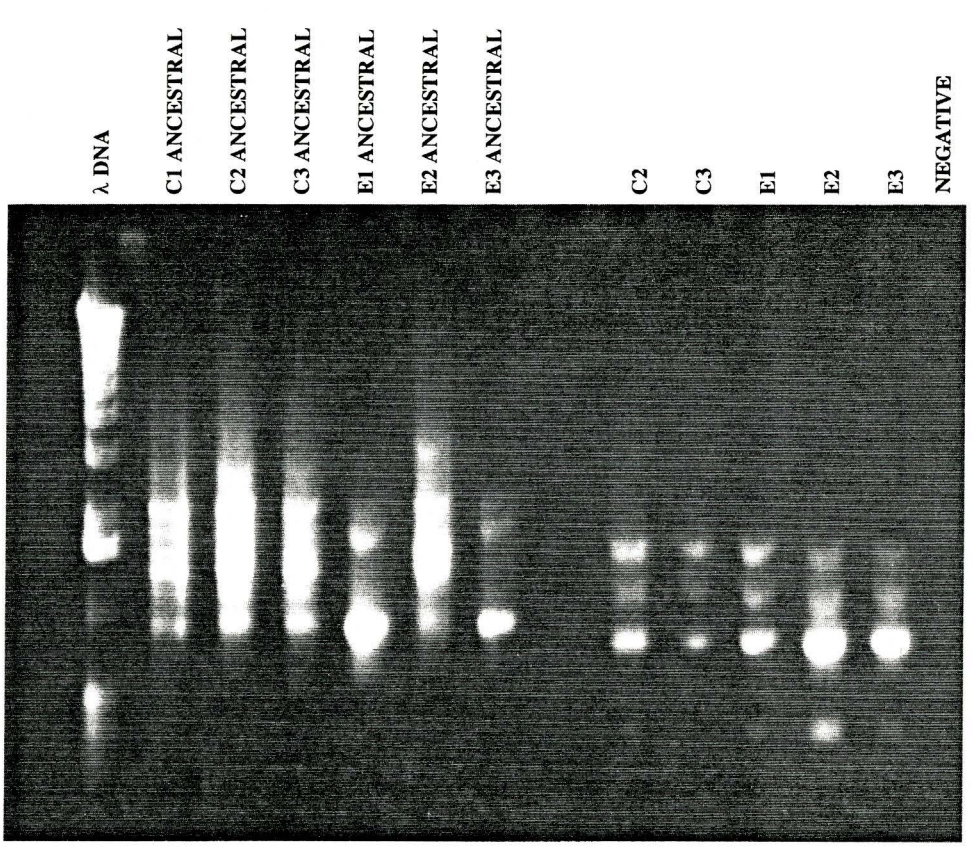
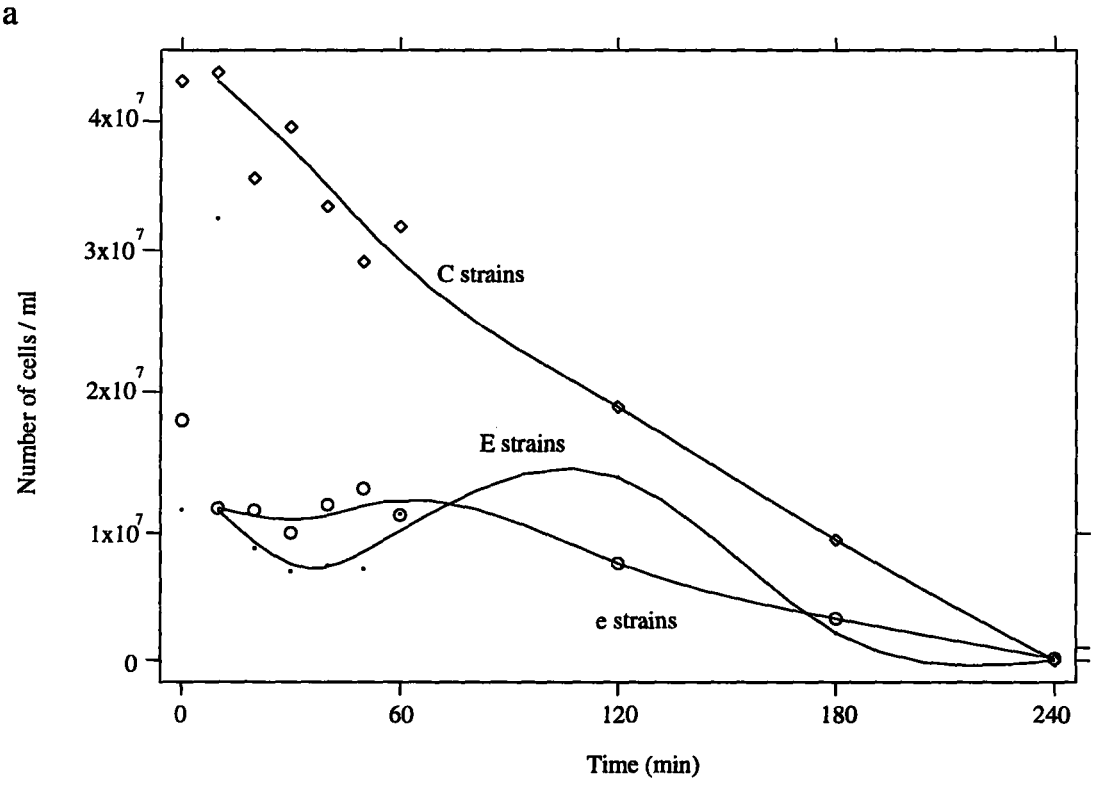
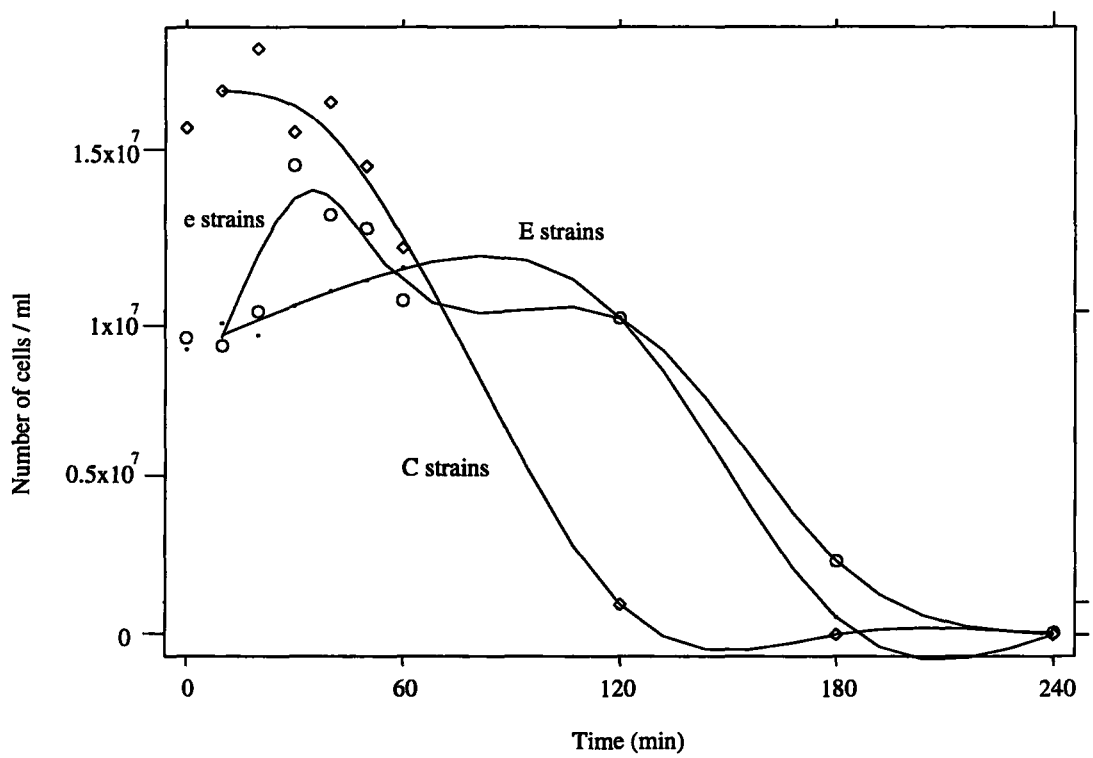

λ DNA
C1 ANCESTRAL
C2 ANCESTRAL
C3 ANCESTRAL
E1 ANCESTRAL
E2 ANCESTRAL
E3 ANCESTRAL
C2
C3
E1
E2
E3
NEGATIVE

Figure 2.5: The effect of a 200μl/10ml challenge dose of EMS on survival of *E. coli* strains (a) "C1", "E1" and "e1", (b) "C2", "E2" and "e2", (c) "C3", "E3" and "e3", over a period of 240 min. Each point represents the average survival of three replicate experiments following EMS challenge. The curves generated are midpoint splines to show general trends in the data and may have greater periodicity than the data warrants.

a


4x10^7
3x10^7
2x10^7
1x10^7
0
Number of cells / ml
C strains
E strains
e strains
0
60
120
180
240
Time (min)

b


1.5x10^7
1x10^7
0.5x10^7
0
Number of cells / ml
e strains
E strains
C strains
0
60
120
180
240
Time (min)

c

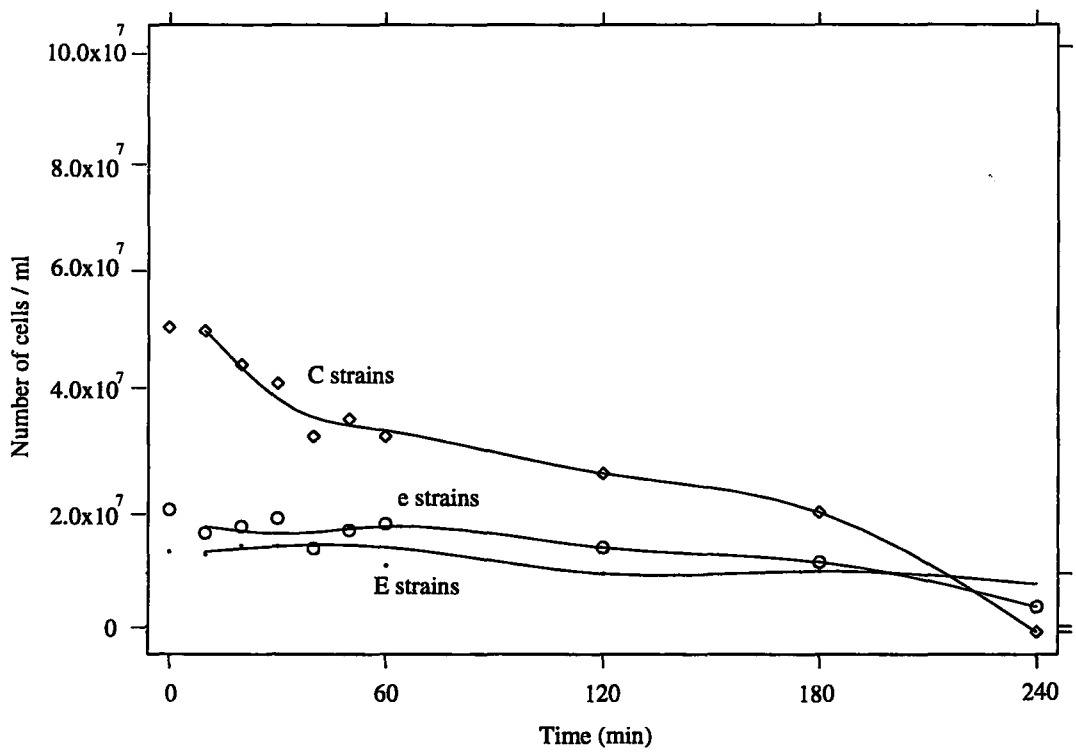
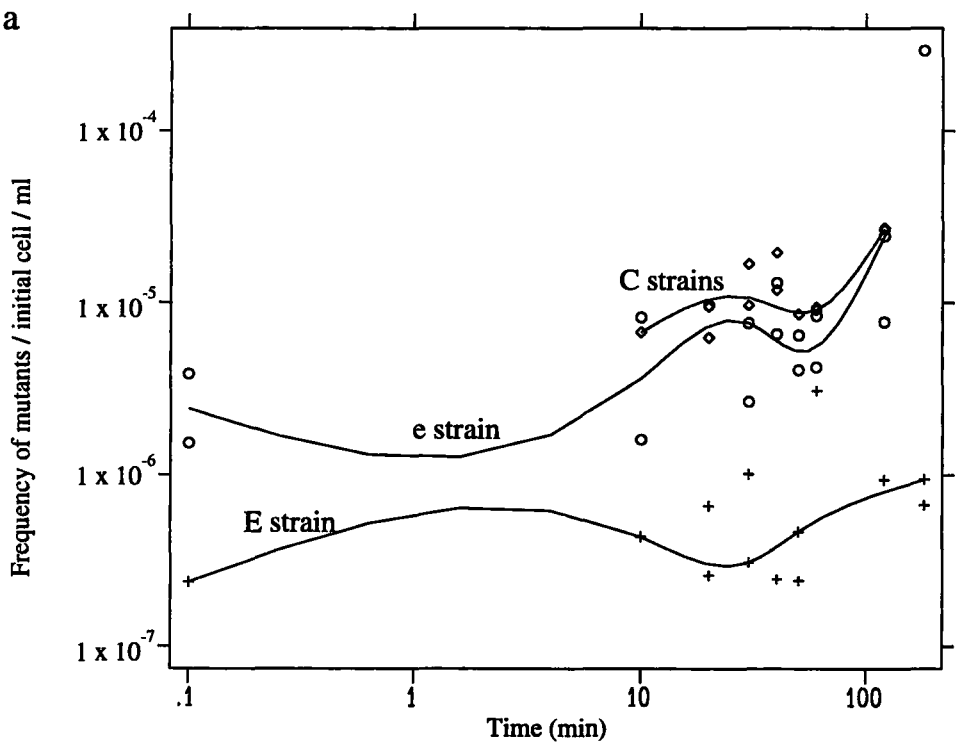

10.0x10^7
8.0x10^7
6.0x10^7
4.0x10^7
2.0x10^7
0
Number of cells / ml
C strains
e strains
E strains
0
60
120
180
240
Time (min)

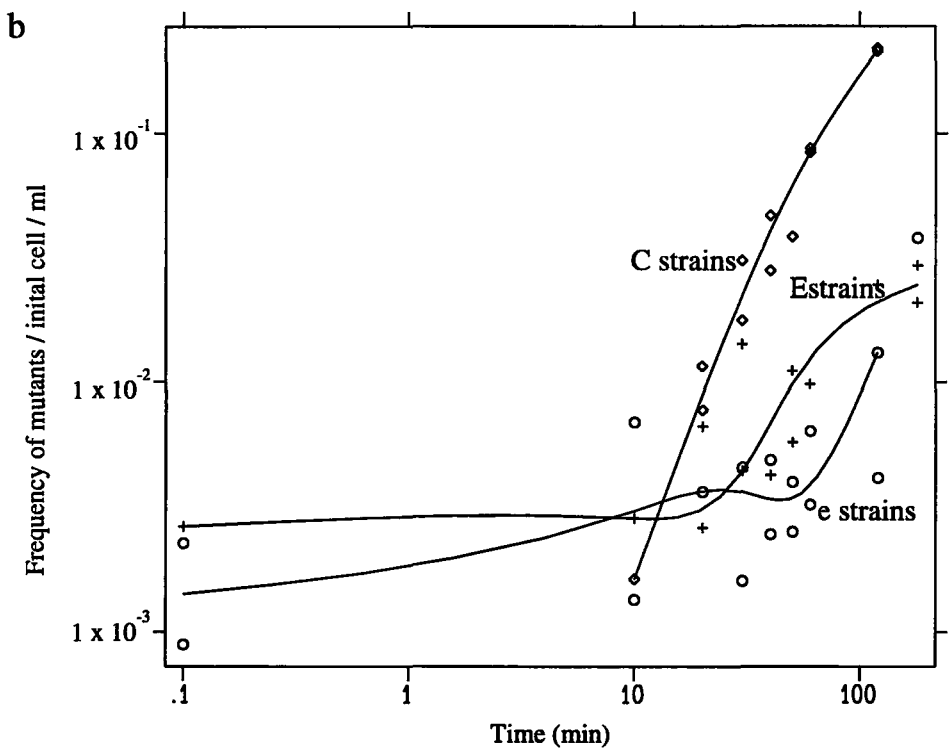
Table 2.4: Lethal Time (LT_{50}) (minutes) measurements following a 200μl/10ml challenge dose of EMS. LT_{50} values were determined by extrapolation from the corresponding survival curves

Cell line	LT_{50} (min)
C_1	96
C_2	78
C_3	173
e_1	147
e_2	163
e_3	235
E_1	166
E_2	152
E_3	> 240

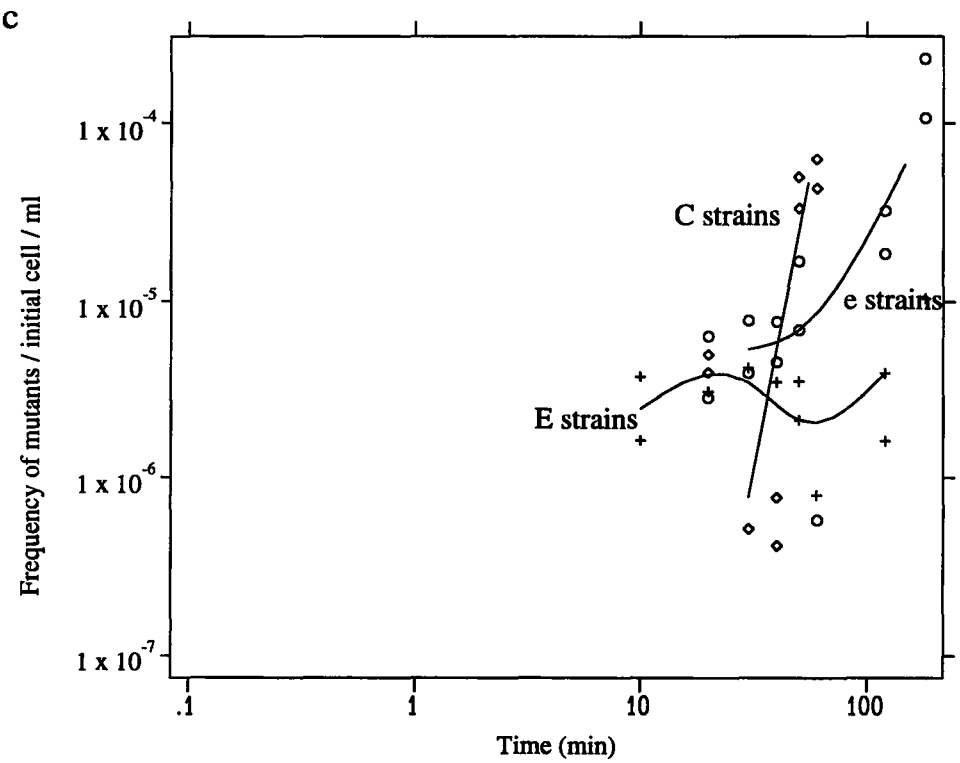
Figure 2.6: The effect of a 200μl/10ml challenge dose of EMS on mutation frequency of *E. coli* strains (a) mutation frequency of "C1", "E1" and "e1" on nalidixic acid plates, (b) mutation frequency of "C1", "E1" and "e1" on streptomycin plates, (c) mutation frequency of "C2", "E2" and "e2" on nalidixic acid plates, (d) mutation frequency of "C2", "E2" and "e2" on streptomycin plates, (e) mutation frequency of "C3", "E3" and "e3" on nalidixic acid plates, (f) mutation frequency of "C3", "E3" and "e3" on streptomycin plates.The curves generated are midpoint splines to show general trends in the data and may have greater periodicity than the data warrants.

a

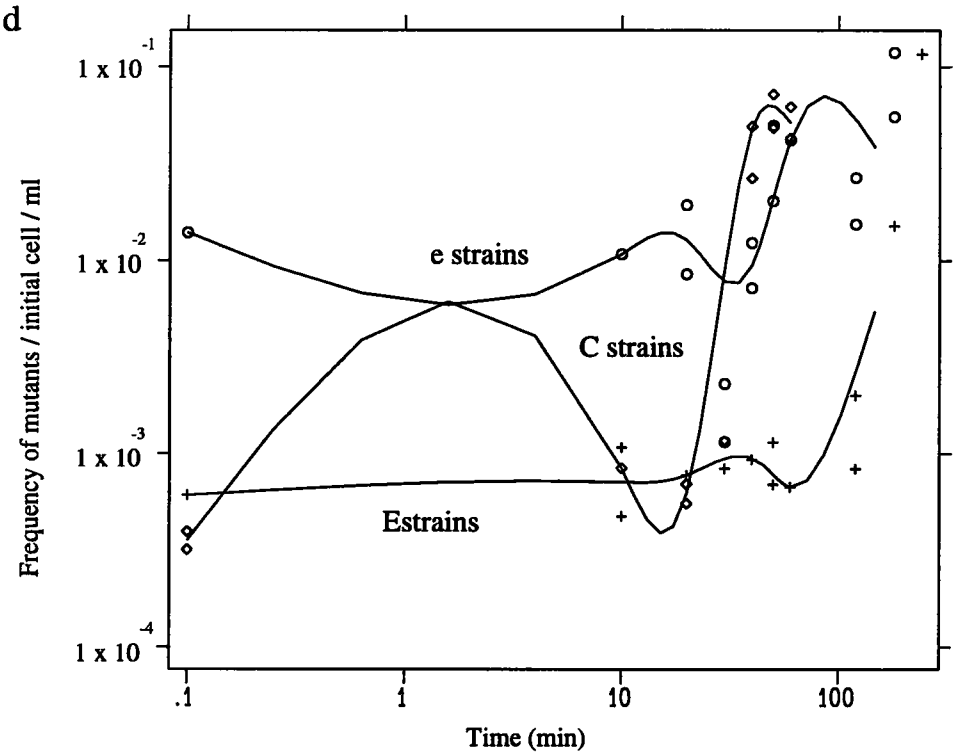

1 x 10^-4
1 x 10^-5
1 x 10^-6
1 x 10^-7
Frequency of mutants / initial cell / ml
C strains
e strain
E strain
.1
1
10
100
Time (min)

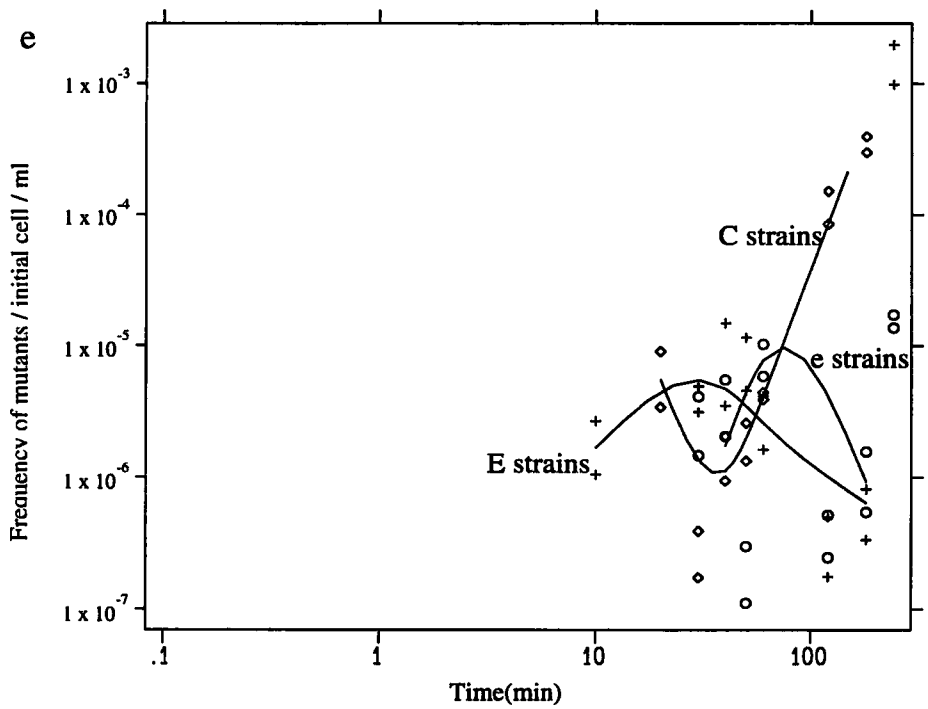

b
1 x 10^-1
1 x 10^-2
1 x 10^-3
Frequency of mutants / inital cell / ml
C strains
Estrains
e strains
.1
1
10
100
Time (min)

c


1 x 10^-4
1 x 10^-5
1 x 10^-6
1 x 10^-7
Frequency of mutants / initial cell / ml
C strains
e strains
E strains
.1
1
10
100
Time (min)

d


Frequency of mutants / initial cell / ml
1 x 10^-1
1 x 10^-2
1 x 10^-3
1 x 10^-4
e strains
C strains
Estrains
.1
1
10
100
Time (min)


e
1 x 10^-3
1 x 10^-4
1 x 10^-5
1 x 10^-6
1 x 10^-7
Frequency of mutants / initial cell / ml
.1
1
10
100
Time(min)
C strains
e strains
E strains

f

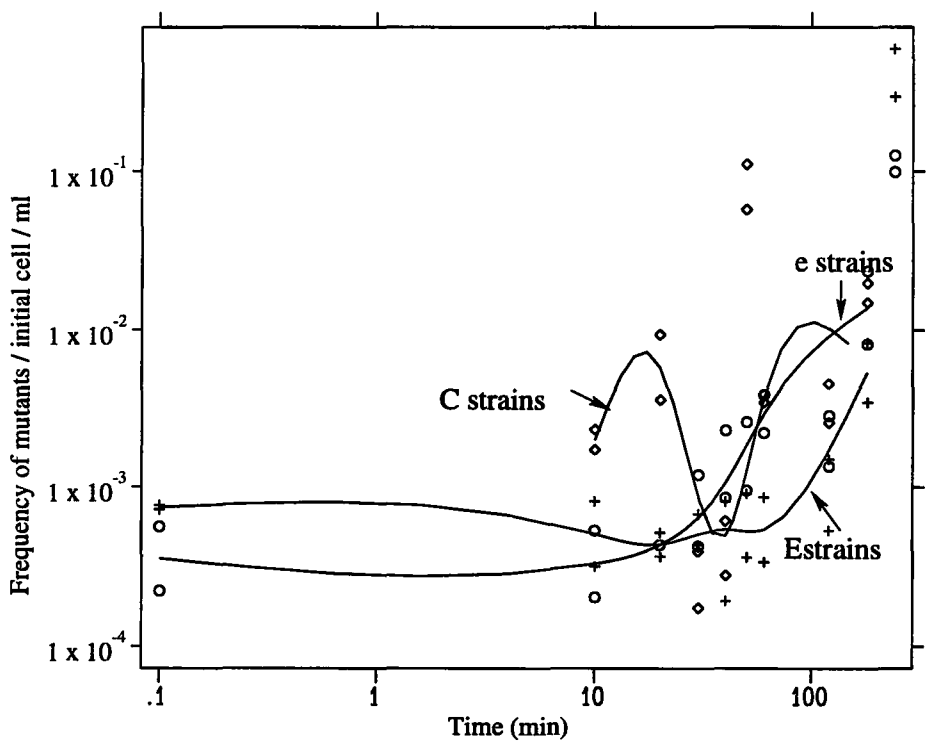
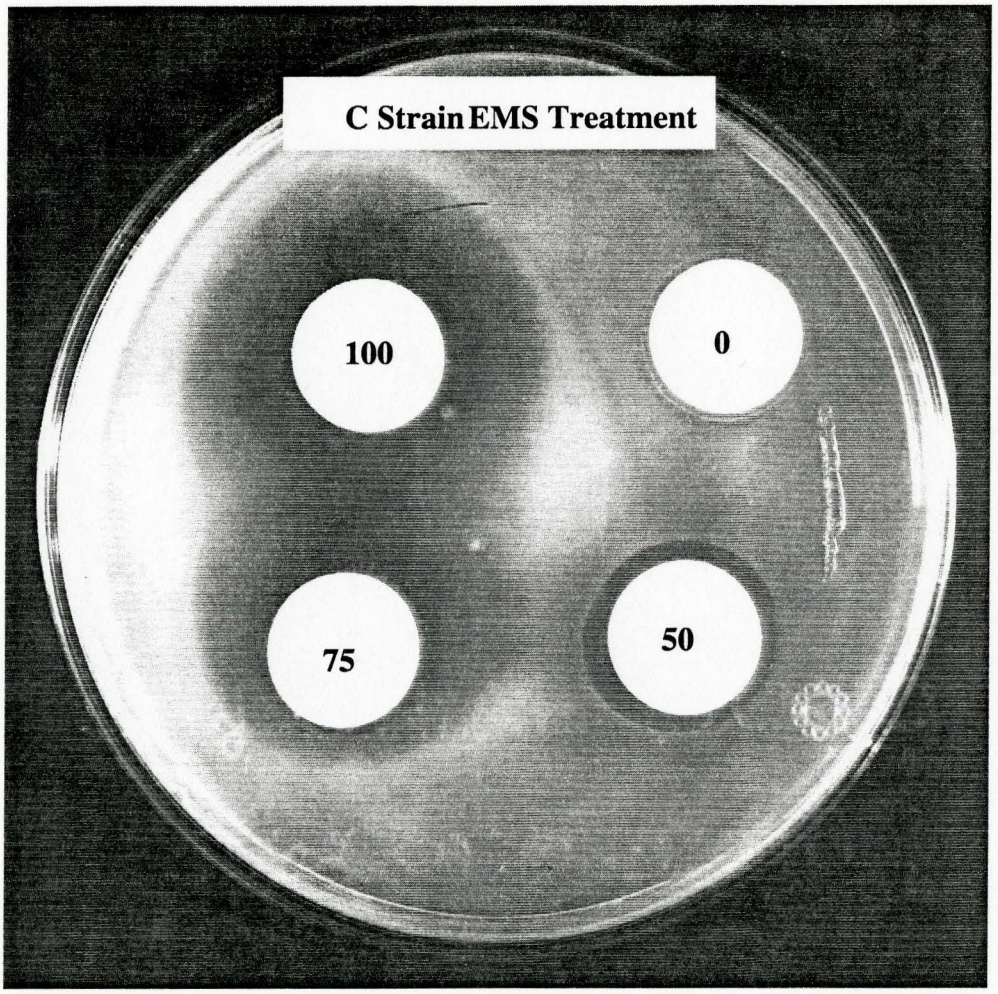
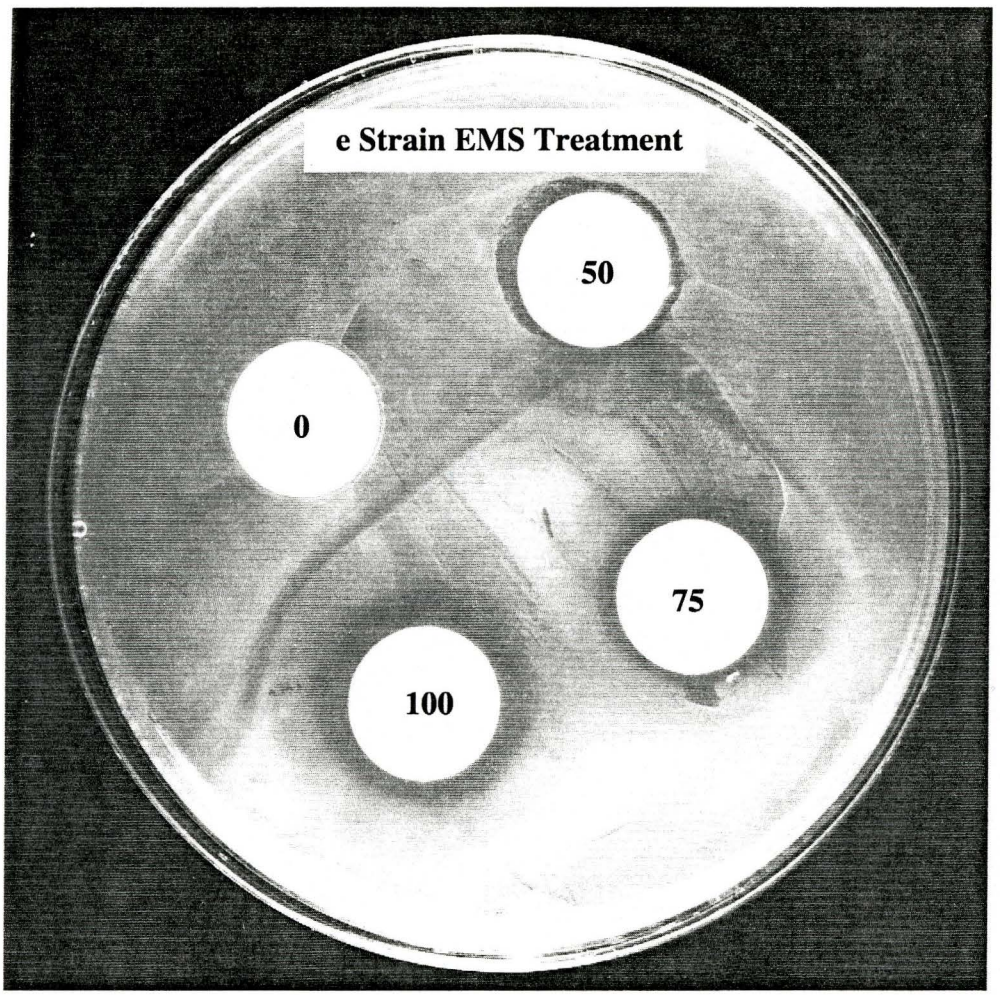

Frequency of mutants / initial cell / ml
1 x 10^-1
1 x 10^-2
1 x 10^-3
1 x 10^-4
.1
1
10
100
Time (min)
C strains
e strains
Estrains

Figure 2.7: Growth inhibition assay with EMS. Cultures were spread over the surface of M9 glucose minimal plates in order to produce bacterial lawns followed by a 2 hour incubation at 37^0C. Three replicate lines of each culture group (a)"C, (b)"e" and (c)"E" were tested. Following incubation, concentrations of 100μl ddH_2O, 50μl EMS/50μl ddH_2O, 75μl EMS/ 25μl ddH_2O and 100 μl of EMS were applied to filter disks and incubated at 37^0C for 48 hours. The distance from the perimeter of the disk to the edge of the halo or point of bacterial growth (zone of inhibition) was measured at five equidistant points.

a



b



c

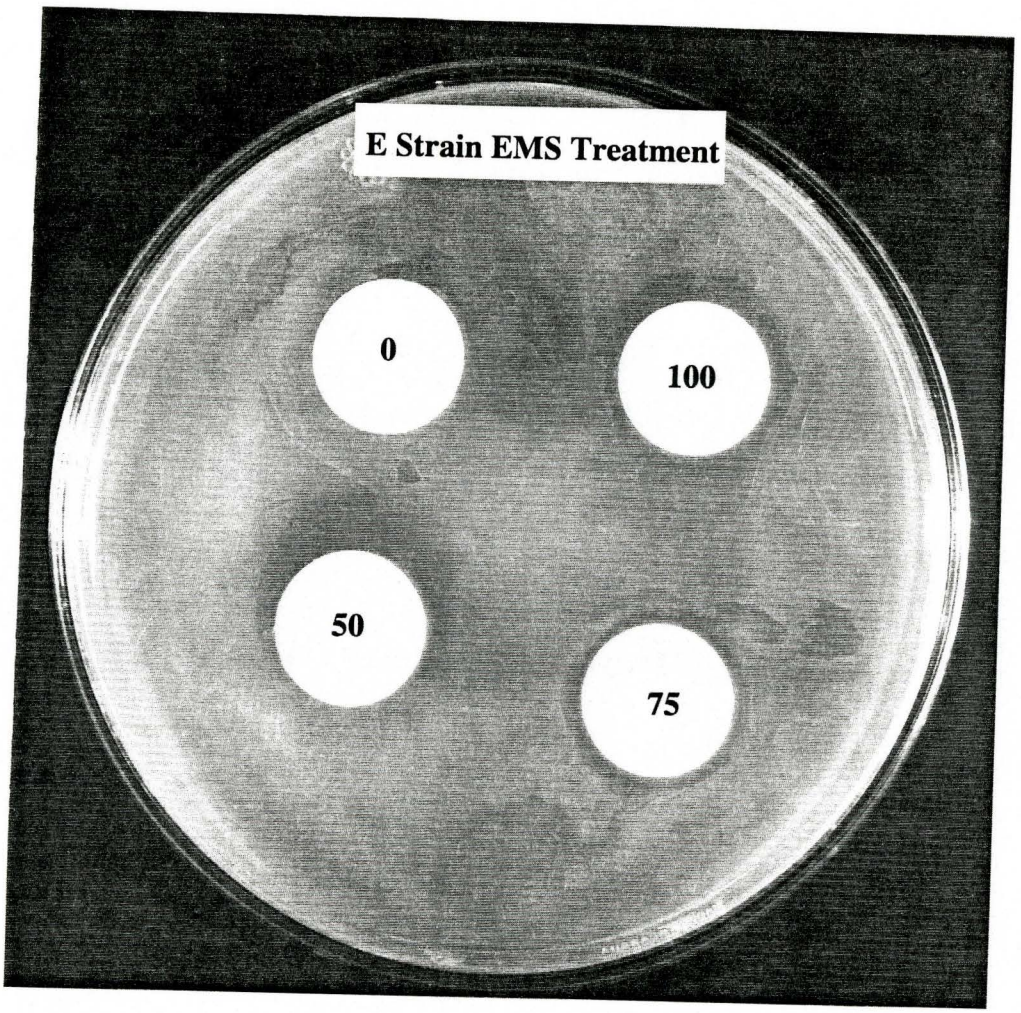


Figure 2.8: Growth inhibition assay with EMS. Cultures were spread over the surface of M9 glucose minimal plates in order to produce bacterial lawns followed by a 2 hour incubation at 37^0C. Three replicate lines of each culture group (a)"C, (b)"e" and (c)"E" were tested on separate plates. Following incubation, concentrations of 100μl ddH_2O, 50μl EMS/50μl ddH_2O, 75μl EMS/ 25μl ddH_2O and 100 μl of EMS were applied to filter disks and incubated at 37^0C for 48 hours. The distance from the perimeter of the disk to the edge of the halo or point of bacterial growth (zone of inhibition) was measured at five equidistant points and averaged for each plate. The values for each replicate plate sampled on the same day were averaged. The mean relative distance were calculated of all three concentrations tested for the adapted "E" and "e" cell lines relative to the control "C" cell lines.

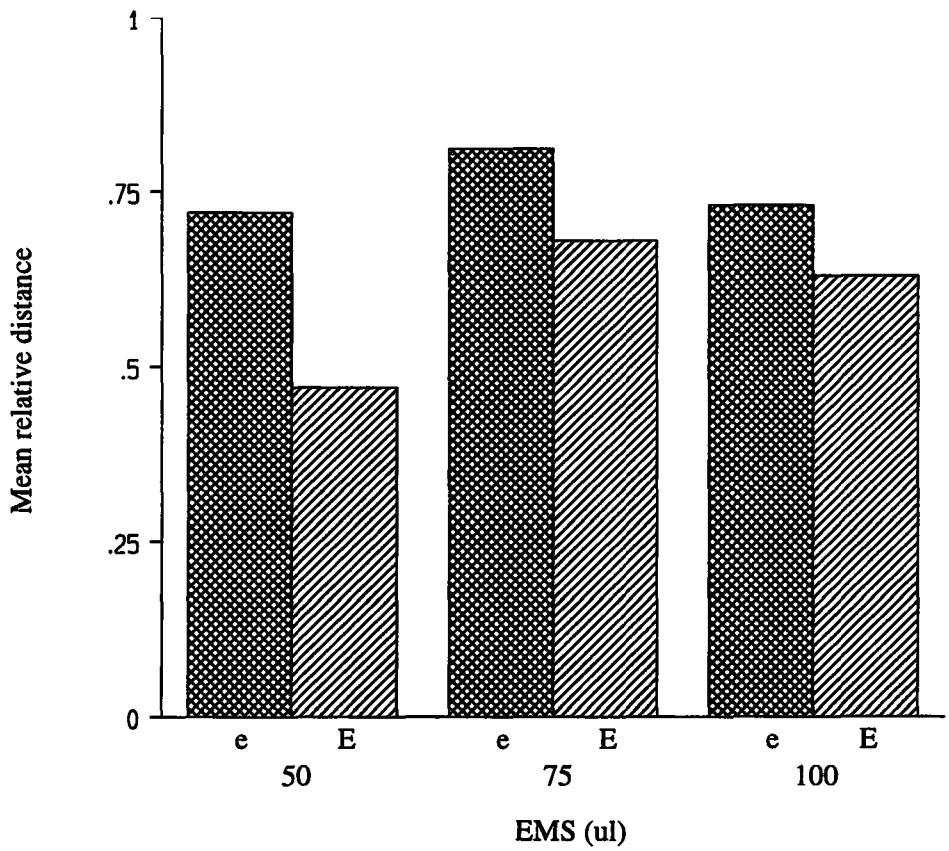
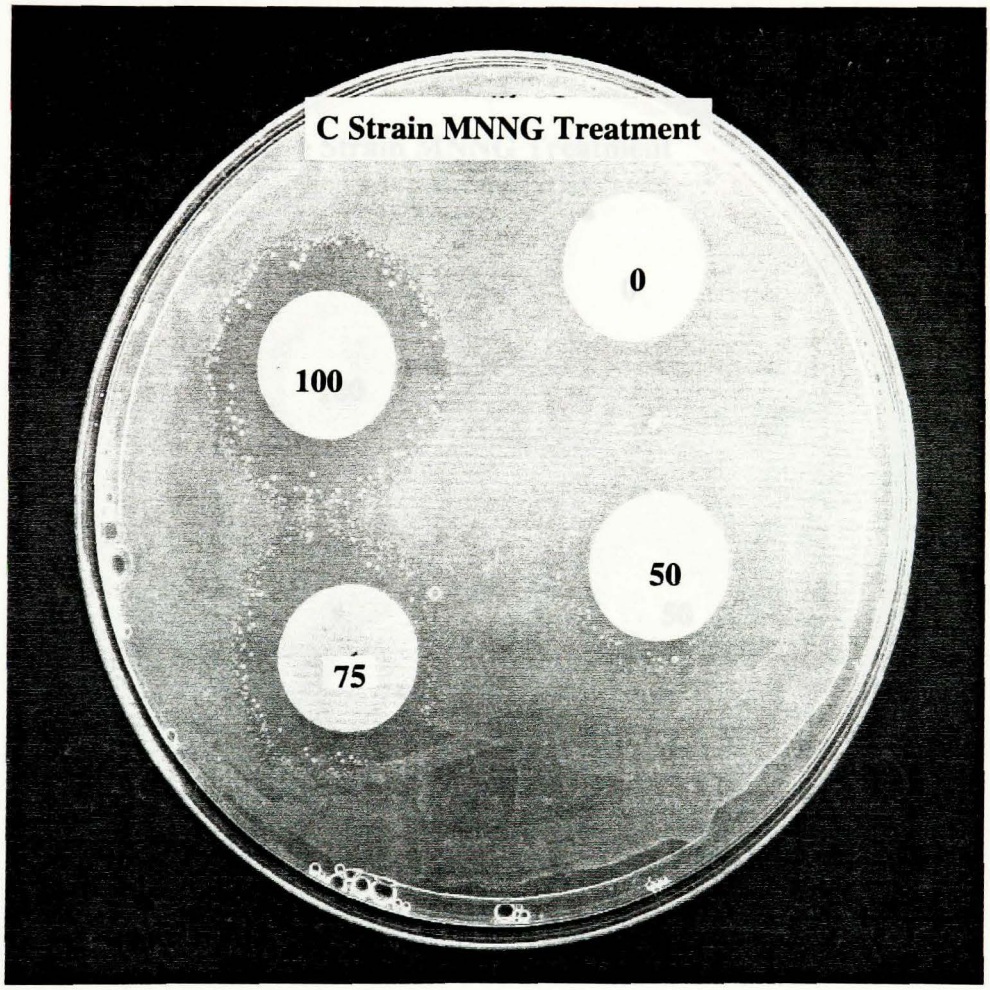
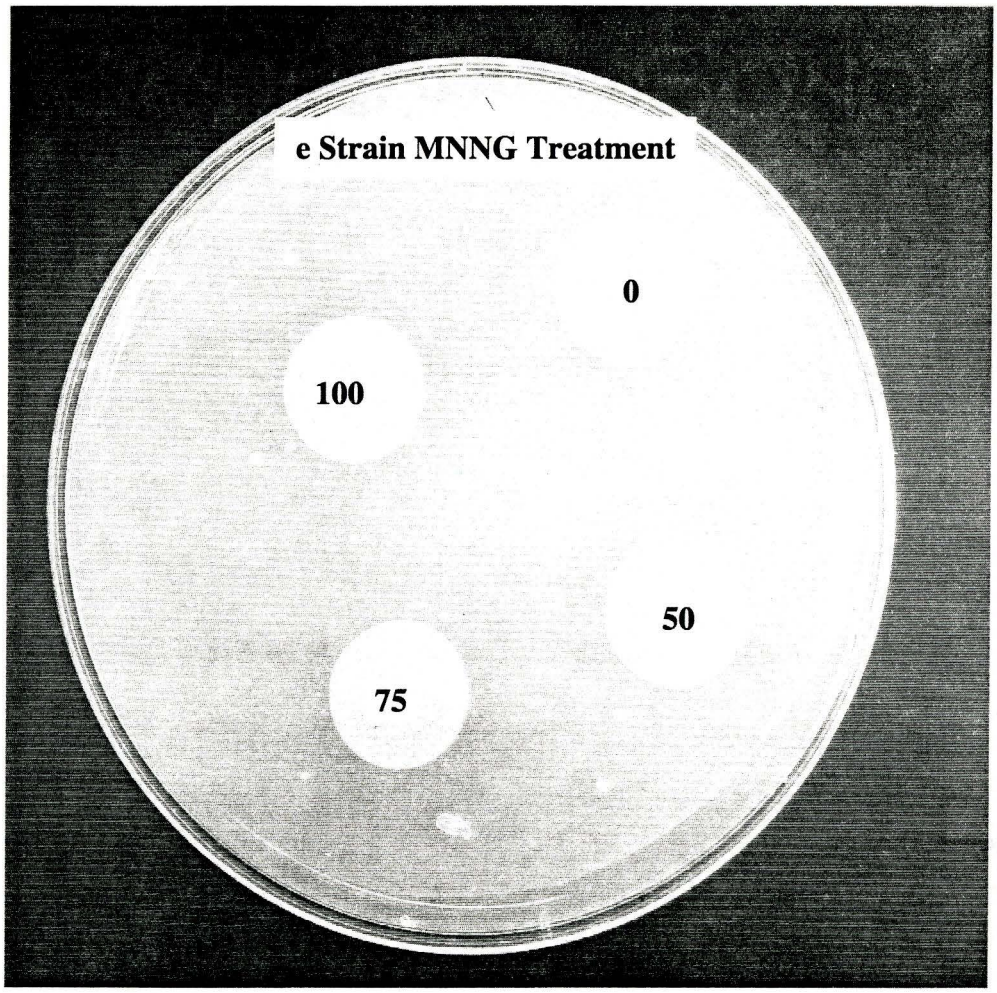

1
.75
.5
.25
0
Mean relative distance
e
E
e
E
e
E
50
75
100
EMS (ul)

Figure 2.9: Growth inhibition assay with MNNG. Cultures were spread over the surface of M9 glucose minimal plates in order to produce bacterial lawns followed by a 2 hour incubation at 37^0C. Three replicate lines of each culture group (a)"C, (b)"e" and (c)"E" were tested. Following incubation, concentrations of 100μl ddH_2O, 50μl of a 1mg/ml MNNG solution/50μl ddH_2O, 75μl of a 1mg/ml MNNG solution/ 25μl ddH_2O and 100 μl of a 1mg/ml solution MNNG were applied to filter disks and incubated at 37^0C for 48 hours. The distance from the perimeter of the disk to the edge of the halo or point of bacterial growth (zone of inhibition) was measured at five equidistant points.

a



b



c

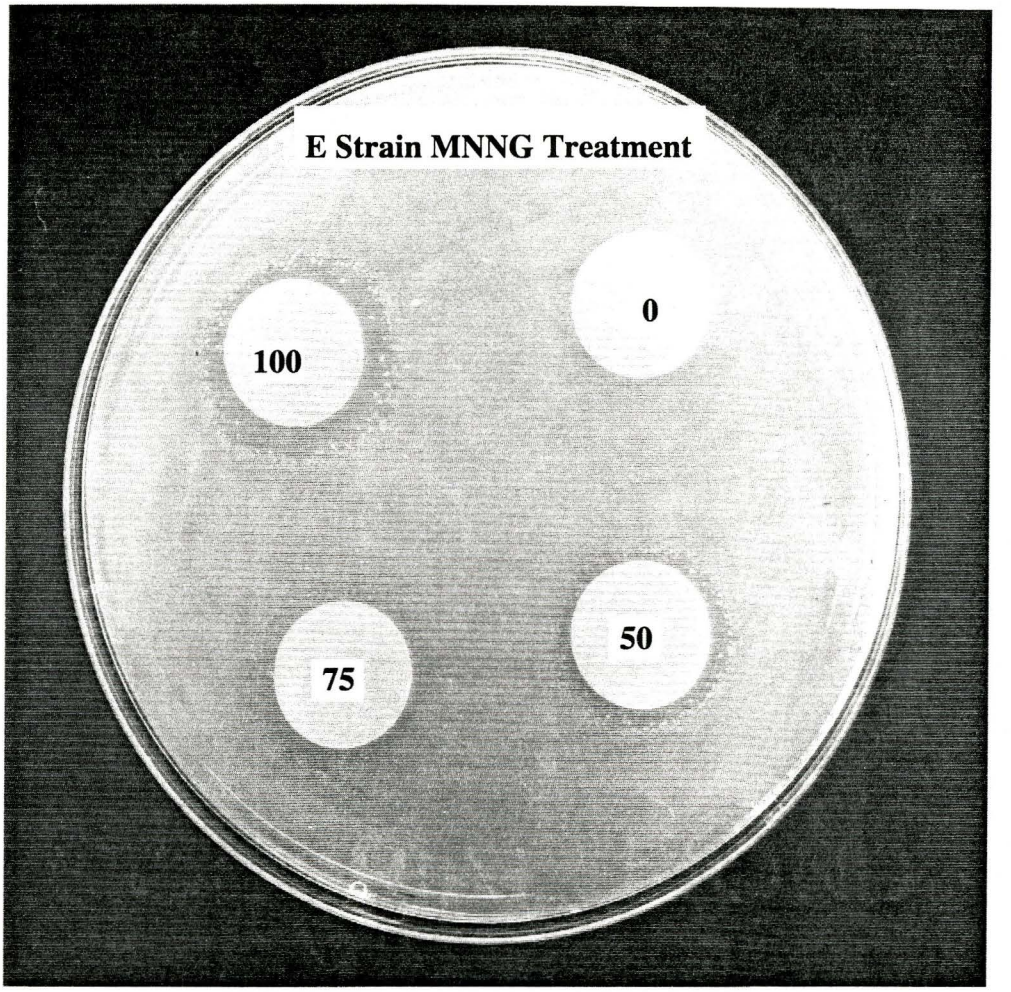
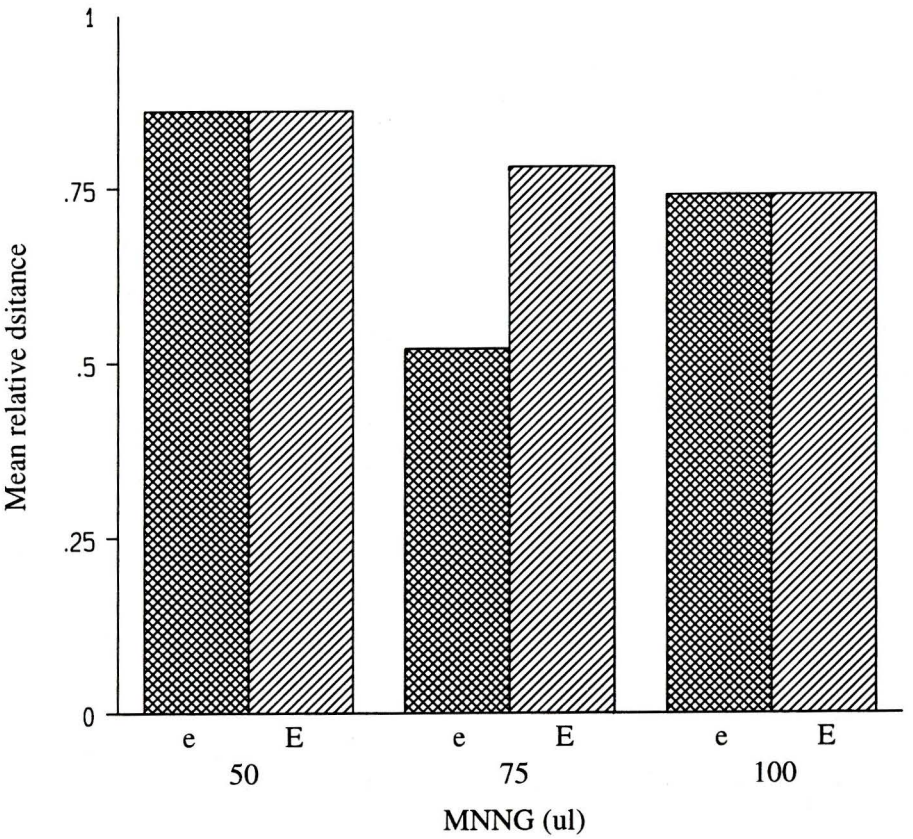


Figure 2.10: Growth inhibition assay with MNNG. Cultures were spread over the surface of M9 glucose minimal plates in order to produce bacterial lawns followed by a 2 hour incubation at 37^0C. Three replicate lines of each culture group (a)"C", (b)"e" and (c)"E" were tested. Following incubation, concentrations of 100μl ddH_2O, 50μl of a 1mg/ml MNNG solution/50μl ddH_2O, 75μl of a 1mg/ml MNNG solution/ 25μl ddH_2O and 100 μl of a 1mg/ml solution MNNG were applied to filter disks and incubated at 37^0C for 48 hours. The distance from the perimeter of the disk to the edge of the halo or point of bacterial growth (zone of inhibition) was measured at five equidistant points and averaged for each plate. The values for each replicate plate sampled on the same day were averaged. The mean relative distance were calculated of all three concentrations tested for the adapted "E" and "e" cell lines relative to the control "C" cell lines.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Chapter 3

Adaptive Response

3.1 Introduction

Organisms are continually exposed to a variety of natural occurring mutagens within the environment and therefore have evolved mechanisms to protect against the toxicity and mutagenicity of these agents (Sedgwick and Vaughan 1991). A significant portion of natural occurring mutagens consist of alkylating agents (Horsfall *et al.* 1990). N-methyl-N'nitrosoguanidine (MNNG) and methanesulfonic acid ethyl ester (EMS) are two powerful alkylating agents often employed in laboratory mutagenesis investigations. These mutagens belong to a class of alkylating agents that react through an SN_1 mechanism and produce primarily mutagenic O^6-alkylguanine lesions (Sledziewska-Gojska and Torzewska 1997) and to a lesser extent the lethal lesions 3-methyladenine and 3-methylguanine (Friedberg, Walker and Wolfram 1995).

In *E. coli* O^6-alkylguanine repair is mediated by two types of alkyltransferases, the Ogt, a constitutively expressed gene product (19-kDa) and the inducible trnsferase Ada (37-kDa) (Roldan-Arjona *et al.* 1994). The 3-methyladenine and 3-methylguanine damage is primarily removed by the constitutive DNA glycosylase I Tag and inducible DNA glycosylae II AlkA which excises a variety of alkylated bases and is directly controlled by the *ada* locus. The adaptive response mechanism was first observed in a study conducted by Samson and Carins in 1977. Upon continuous exposure of *E. coli* to low doses of the alkylating agent MNNG (N-methyl-N'nitro-N-nitrosoguanidine), *E. coli* cells develop significant resistance to the mutagenic and lethal effects of higher challenge doses of MNNG. This inducible repair mechanism is distinct from UV-induced error-prone SOS repair since it is error-free allowing increased survival at no mutational cost to the cell and a significantly reduced mutation frequency compared to non-adapted cells (Lindahl *et al.* 1988; Samson and Cairns 1977). Studies indicate this repair process to be independent of *recA* and *lexA* genes and not inducible by UV or 4-nitroquinoline 1-oxide (Jeggo *et al.* 1977; Schendel *et al.* 1978). In *E. coli* the inducible O^6-methylguanine-DNA methyltransferase I (O^6MGT I) or Ada protein acts to reduce alkylation damage by transferring the methyl group from the alkylated DNA to its own cysteine residue, leaving behind an unmodified guanine base (Demple *et al.* 1982; Lindahl *et al.* 1988). The methylated Ada molecule is quite stable and not actively demethylated and as a result is inactivated and expended in each reaction (Friedberg, Walker and Wolfram 1995). Another component of the adaptive response is the inducible DNA-glycosylase II or AlkA protein. This enzyme is also controlled by the *ada* locus. It removes 3-methyladenine and 3-methylguanine damage remaining within the minor groove by excision repair

and requires a functional DNA polI for gap filling.

All of the studies to date investigating adaptation to alkylating damage have primarily observed *E. coli's* ability to acclimate phenotypically over short time intervals to EMS in order to induce the adaptive response repair pathway. However, it is impossible to observe the effects of mutagenic agents on organisms and in turn populations in such short term analysis, since new mutations may not necessarily contribute extensively to genetic variation relative to that which is already present. In previous studies long-term adapted cell lines continually exposed to low doses of the alkylating agent Ethyl methane sulfonate (EMS) for over 3000 generations cell lines have demonstrated significantly enhanced resistance to the lethal effects and a higher tolerance to the mutagenic effects of large challenge doses of EMS (section 2). In this study we attempt to induce the adaptive response mechanism in these same long-term genetically adapted cell lines to investigate if it is possible to induce the adaptive response repair pathway in these cell lines and further enhance the level of resistance.

3.2 Methods

Bacterial Lines. The experiments were conducted using cell lines from an ancestral *E. coli* CSH100 [*ara* Δ (*gpt-lac*)5 mating type F′ *lacpro* A^+ B^+] clone obtained from Cold Spring Harbour Laboratory. This strain was separated into six individual lines that were then maintained by serial culture as pure lines for over 10 months. At this point in time these ancestral cell lines ("generation 0") were further propagated for an

additional 24 months (over 4000 generations) for long-term adaptation experimental analysis.

Culture Conditions. Three replicates of two experimental treatment lines were established and have been maintained for approximately 3 years at 37^0C. All lines were propagated with M9 minimal medium and maintained daily by 1:100 serial dilution into 10ml of fresh medium. The EMS adapted lines designated as "E" were treated daily with 25 μl of EMS following serial dilution. The control lines designated as "C" were grown in the absence of EMS. All other conditions were identical for both the "E" and the "C" lines. In most of the experiments conducted a third cell line was established using an inoculate from the "E" EMS adapted line. This line designated "e", was grown up overnight (approximately 8 generations) in the absence of EMS in order to eliminate any physiological changes and to distinguish between phenotypic acclimation and genetic modification.

Chemical Reagents. Ethyl methane sulfonate (EMS), N-methyl-N'nitroso-guanidine (MNNG), agar and all necessary supplements were obtained from Sigma Chemical Co.

Bacterial Growth Medium. Liquid growth media consisted of minimal M9 solution supplemented with 20% glucose, 1M $MgSO_4$, and 0.5% thiamine per litre.

Several experiments were conducted in an attempt to induce the adaptive response repair mechanism in our "E" and "C" *E. coli* cell lines. Two cultures were generated for each line, the adapted and the non-adapted. Adaptation in this section is used to describe short term mutagen treatment applied to the cell lines immediately prior to

application of a challenge dose rather than the long-term exposure of the "E" lines to EMS. "E", "e" and "C" cultures were generated (as described previously).

A total of six 10ml cultures, two for each line designated adapted and non-adapted, were generated from 500 μl inoculates from "C", "e" and "E" overnight cultures. 25 μl of EMS was added to the "E" cultures. All six cultures were grown to log phase. During this time the adapted cultures received a dose 2μl or 0.2μl of EMS per 10ml culture for 90 min, while the non-adapted cultures received no treatment. Following this adaptation all cultures were challenged with a 200μl dose of EMS per 10 ml culture. Samples were taken from all six cultures every ten minutes for the first hour and thereafter every hour for an additional 3 hours. Samples were then appropriately diluted and plated for survivors. Overnight cultures were generated by diluting samples 1:100 in fresh LB and growing overnight at 37^0C. The following day samples were plated on LB+rifampicin and LB+naladixic acid plates to determine the frequency of $Rif^{(r)}$ and $Nal^{(r)}$ mutants respectively. All plates were scored after 18-24h at 37^0C.

The above experiment was replicated using a 1μg μ/l adaptive and 100μg μ/l challenge dose of MNNG(N-methyl-N'nitroso-guanidine). All of the cell lines and the experimental condition were identical to that used in the EMS adaptive response experiment described above.

3.3 Results

Figures 3.1 (a), (b) and (c) display the survival response of the cell lines following a 2μl challenge dose of EMS. The lines are graphed in pairs in order to observe the differences of a cell line relative to it adapted counterpart. Both "C" and "$C^{adapted}$" lines appear to have almost identical survival responses to the challenge dose of EMS with a relatively constant survivorship at approximately $4x10^6$ cells/ml (Figure 3.1(a)). At 180 minutes the "$C^{adapted}$" cell line appears to decline slightly in cell numbers compared to the "C" line with approximately $2x10^5$ and $3x10^6$ cells respectively. The "e" and "$e^{adapted}$" cell lines also demonstrate similar survival until approximately 180 minutes at which time there is a significant decline in cell survival to a point of complete cell death at 240 minutes while the "e" strain retains a count of approximately $1x10^5$ cells (Figure 3.1(b)). This pattern is also observed with the "E" and "$E^{adapted}$" cell lines showing no significant difference in cell survival till approximately 180 minutes at which time the "$E^{adapted}$" strain reaches cell death and the "E" line indicates cell viability of approximately $5x10^5$ cells. The mutation frequency analysis of these cell lines on nalidixic acid plates indicates a similar pattern of mutation frequency for the cells "C", "e" and "E" relative to their adapted counterparts "$C^{adapted}$", "$E^{adapted}$" and "$E^{adapted}$" (Figures 3.1 (d), (e) and (f). The "E" and "e" cell lines appear to have a relatively constant mutation frequency of $5x10^{-3}$ and $1x10^{-3}$ respectively until complete cell death was reached. The "C" cell line however showed a steady accumulation of mutants with an initial average mutation frequency of approximately $1x10^{-5}$ and a final average mutation frequency for the two cell lines at approximately $1x10^{-2}$.

Mutation frequency analysis on rifampicin plates shows a similar pattern for the "E" and "e" cell lines relative to the nalidixic acid plates (Figures 3.1 (h) and (i)). In both graphs the "e" and "E" cell lines a similar mutation frequency pattern to their adapted counterparts "$e^{adapted}$" and "$E^{adapted}$". There also appears to be relatively little change in mutation frequency over the initial 180 minutes following exposure to the challenge dose of EMS. Again, similar to the nalidixic acid assay the "C" and "$C^{adapted}$" lines appear to incur a larger increase of mutation as a result of the challenge dose of EMS. In this experiment the "$C^{adapted}$" strain appears to have a significantly larger increase in mutation frequency from 120 to 240 minutes following the EMS challenge resulting in a frequency of $1x10^{-2}$ mutants compared to the "C" line which has a final frequency of $6x10^{-4}$ mutants. A reduced EMS adaptive dose was used in an attempt to induce the response. The survivorship of the "C", "e" and "E" cell lined produces almost identical responses to their adapted counterparts "$C^{adapted}$", "$e^{adapted}$" and "$E^{adapted}$" (Figures 3.2 (a), (b) and (c)). Similar results were observed for the mutation frequency assay when tested on nalidixic acid (Figures 3.2 (d), (e) and (f) and rifampicin (Figures 3.2 (g), (h), (i)). The mutation frequency appeared to remain relatively constant for "E" cell lines when tested on nalidixic acid and rifampicin remaining at approximately $1x10^{-3}$ and $5x1^{-4}$ respectively. This was also observed for the "e" cell line when tested on rifampicin, however a continuous mutation frequency increase is observed when tested on nalidixic acid. The "C" cell lines result in a continuous increase in mutants for both nalidixic acid and rifampicin from approximately $5x10^{-5}$ (initial) to $5x10^{-4}$ (60 min) and $5x10^{-5}$ (initial) to $5x10^{-1}$ (180 min) respectively.

Adaptive response assays conducted with MNNG resulted in very similar responses in mutation frequency to the observations when cells were challenged with EMS. Initially the cells were tested for survivorship following a 100μl challenge dose of MNNG (Figure 3.3). These results indicate that there was no difference between the "C", "e" and "E" cell lines relative to their adapted counterparts "$C^{adapted}$", "$e^{adapted}$" and "$E^{adapted}$". Both the and "E" cell lines declined in their survivorship very slowly over he course of 15 minutes from approximately $7x10^6$ to approximately $7x10^5$ and from approximately $3x10^6$ to approximately $2x10^5$ over the 15 minute sampling period. This was not the case for both the "C" and "$C^{adapted}$" cell lines. These cell lines began to decline almost immediately after initial MNNG challenge from approximately $8x10^6$ (initial) to approximately 1 x 10^2 cells (15 min) which is significantly faster than either the "e" or "E" cell lines. A replicate experiment resulted in identical survival responses (Figure 3.4 (a)). LT_{50} values were determined for each cell line by extrapolation from the corresponding survival curves. This value is a measurement of the time it takes for the cell line to be reduced to one half of its starting cell density upon exposure to mutagen treatment. These values are listed in table 3.1 and 3.2.. The Lethal Time values sugges that the "E" cell line has the highest tolerance to the challenge mutagen for survival curve Figure 3.3 and "C" the highest tolerance to the challenge mutagen for survival curve Figure 3.4. Although these LT_{50} values suggest immediate sensitivity levels of the cell lines to the toxic effects of the challenge mutgen, these values do not give an indication of the overall tolerance of cell lines since most of the decline in cell surviorship begins approximately between 5-6 minutes following exposure to the challenge dose for the the control "C" and both the adapted "E"/"e" cell lines. Following the start of this phase of rapid

decline in survivorship it is obvious in both survival curves (Figures 3.3 and 3.4) that the adapted cell lines"E"/"e" have enhanced levels of resistance to the toxic effects of the challenge mutagen and these cell lines survive beyond the sampling periods while the control cell lines have very low viable cell counts by the final 15 minute sampling period.

Mutation frequency analysis on kanamycin plates indicate no significant differences in mutation frequency between "C", "e" and "E" cell lines relative to their adapted "$C^{adapted}$", "$e^{adapted}$" and "$E^{adapted}$" counterparts (Figure 3.4 (b)). The "C" and "$C^{adapted}$" lines appeared to have a significantly higher initial mutation frequency (approximately $1x10^{-2}$) than either the "e" or "E" cell lines. Despite the higher initial frequency all three groups appear to incur similar increases in mutants over the course of the 15 min sampling period. These results are similar to mutation frequency analysis on nalidixic acid plates (Figure 3.4(c)). The mutation frequency begins slightly higher for the two "C" groups compared to the "e" and "E" lines and appears to increase more rapidly compared to either the "e" or the "E" cell lines. Both the "e" and the "E" cell lines result in almost identical responses compared to their adapted counterparts "$e^{adapted}$" and "$E^{adapted}$". This is also the case for the "C" cell lines however at approximately 10minutes following challenge the "$C^{adapted}$" cell lines appears to significantly decrease in the number of nalidixic mutants while its "C" counterpart continues to increase resulting in mutation frequencies of $1x10^{-4}$ and $5x10^{1}$.

3.4 Discussion

The observed results in this investigation indicate an inability to induce the adaptive response repair mechanism in any of our three cell lines "C", "e" or "E". On the whole there were no significant differences observed in the cells relative to their adapted counterparts in either the survival curves or in mutation frequency analysis. The initial $2\mu l$ adaptive dose of EMS used in an attempt to induce the adaptive response in the long-term evolved cell lines is the identical concentration shown to induce the adaptive response in a previous study (Schendel *et al.* 1978). A lower adaptive dose of $0.2\mu l$ was also unsuccessful at reducing the lethal and mutagenic effects of the large EMS challenge dose.

Studies have shown that EMS is not proficient at inducing the adaptive response when tested in the K-12 strain AB1157 yet some degree of mutagenic and lethal adaptation was observed in other studies using F-26 a B/r strain (Jeggo 1980) suggesting that the induction may be strain sensitive. Although the adaptive response has shown to be illicited by and act upon EMS lesions in some strains, it is not believed to be a strong inducer of the adaptive response since the lesions produced by this mutagen are not located at the replication fork which is the preferred location for O^6-DNA-methyl transferase activity (Jeggo *et al.* 1977). MNNG has shown strong induction of the adaptive response in most strains tested. I attempted to induce the response using MNNG as the adaptation and challenge mutagen in the long-term evolved lines in order to see if the inability to induce the adaptive response is a result of the mutagen being used. However, the adaptive response could not be induced using this strong

adaptive response inducer suggesting the possibility that the adaptive concentrations were insufficient or an inability to induce the response in the cell lines themselves. This differential induction of the adaptive response in different *E. coli* strains has been observed in previous studies (Jeggo 1980).

In most cases the ability or inability to induce mutagenic and lethal adaptation always occurs in parallel suggesting that this phenomenon is most likely a consequence of inhibition of transcription activation of the *ada* gene. *Salmonella typhimurium* studies indicate an inability to induce the adaptive response as a result of weak transcriptional activation of its *ada* gene due to self methylation of the protein during pre-treatment with MNNG (Vaughan and Sedgwick 1991). Investigators have discovered that the Ogt protein rather than the Ada protein protects *S. typhimurium* from the mutagenic damage caused by alkylating agent (Yamada *et al.* 1995). The self methylation of the Ada protein is also responsible for the inability to induce the "Adaptive response" when pre-adapted and challenged with SN_2 alkylating agents MMS and DMS (Sledziewska-Gojska 1995). Studies indicate that the primary control mechanism of Ada activation is the levels of unmethylated Ada. Levels above 200-500 unmethylated Ada molecules per cell appear to result in inhibition of *ada* transcription, which is not observed in cells containing identical levels of methylated Ada molecules (Saget and Walker 1994). These observations suggest that Ada may negatively modulate its expression. It is suggested that the induction of the response and the level of sensitivity of cells to the alkylating agent is dependent upon a variety of factors. In addition to unmethylated Ada levels factors such as strain, mode of alkylation damage, cellular content of methylguanine methyl transeferase, and non-

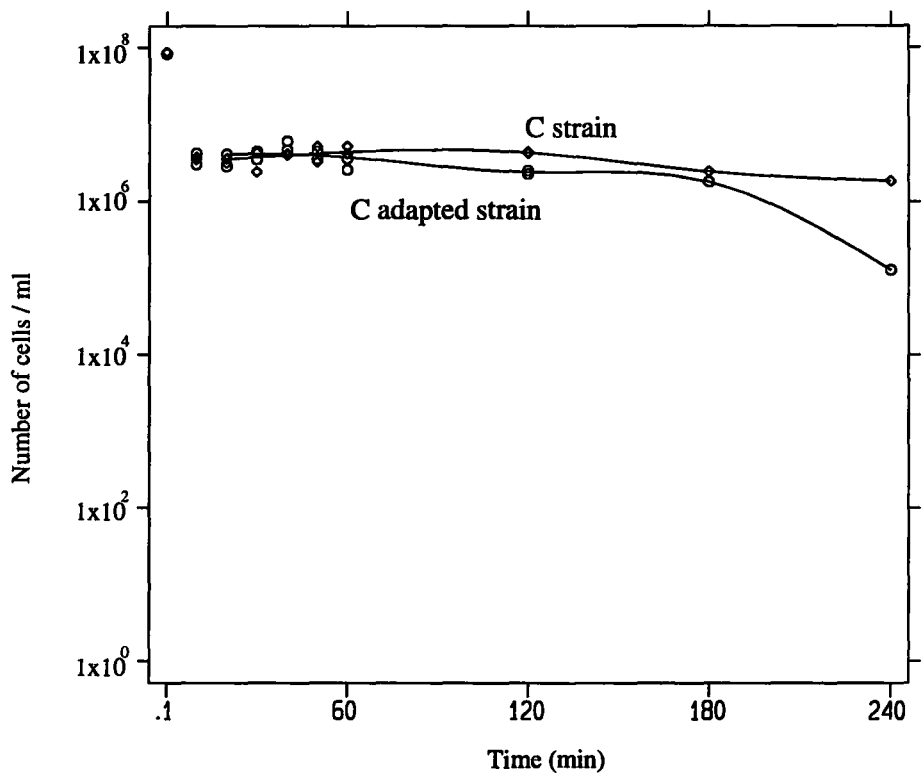
specific induction can affect the ability of cells to induce the Adaptive response when challenged with a large dose of alkylating agent. The Ada protein binds to a promoter region of the *ada/alkb* and *alka* genes. The binding sites of the *ada/alkb* and *alk* genes contain a common promoter region (AAANNAAAGCGCA) termed the "Ada Box" (Lindahl *et al.* 1988; Nakamura *et al.* 1988; Landini and Volkert 1995; Friedberg, Walker and Wolfram 1995). Mutations in this sequence eliminate transcriptional activation of the *ada* gene (Nakamura *et al.* 1988; Landini and Volkert 1995). It is possible that any one of these factors may be involved in the inability to induce the response in the long-term evolved lines.

Despite the observation that survival curves of the MNNG challenged cell lines do not indicate the induction of the adaptive response in the short term adapted cell lines, there is a significantly enhanced resistance to the lethal effects of the challenge MNNG in the "E", "E adapted", "e" and "e adapted" cell lines indicating a genetic resistance to the mutagen even in the absence of physiological induction of the "Adaptive response". The MNNG survival curves indicate identical cell line survival responses and slightly reduced mutation frequencies for the long-term EMS adapted cell lines as observed with EMS challenge (as described in Section 2). These observations suggest a cross adaptive response between EMS and MNNG and further support the findings from the MNNG disk assay analysis showing reduced growth inhibition zones in cells adapted to EMS and challenged with MNNG (as described in Section 2). Cross adaptation has been observed with preadaptaion to nonalkylating mutagens such hydrogen peroxide. High doses of hydrogen peroxide results in cellular adaptation to the lethal effects of MNNG. However the cross adaptive effects is time

dependent and begins to decrease at approximately 15 min after pretreatment with hydrogen peroxide and lasts approximately 30 minutes (Asad *et al.* 1997). The observations of this analysis indicate an inability to induce the adaptive response in the long-term evolved "C", "e" or "E" cell lines with low doses of either EMS or MNNG. Genetic adaptation resulting in increased resistance to the lethal effects of MNNG is observed in the long-term EMS adapted cell lines "e" and "E" support previous EMS studies with these lines. This enhanced resistance could likely be a result of enhanced activity of the constitutive transferase Ogt and the constitutive glycosylase Tag; the two enzymes responsible for the majority of the repair of O^6-methylguanine and 3-methyladenine/3-methylguanine respectively in organisms unable to induce the adaptive response (Yamada *et al.* 1995).

Figure 3.1: The effect of a $100\mu l/10ml$ challenge dose of EMS on survival and mutation frequency of *E. coli* cell lines "C" and "$C^{adapted}$", "e" and "e ^{adapted}", "E" and "E ^{adapted}". Adapted strains received a $2\mu l/10ml$ adaptive dose for 90 min prior to challenge dose. (a-c) survival on LB media, (d-f) mutation frequency on nalidixic plates, (g-i) mutation frequency on rifampicin acid plates.The curves generated are midpoint splines to show general trends in the data and may have greater periodicity than the data warrants.

a


1x10^8
1x10^6
1x10^4
1x10^2
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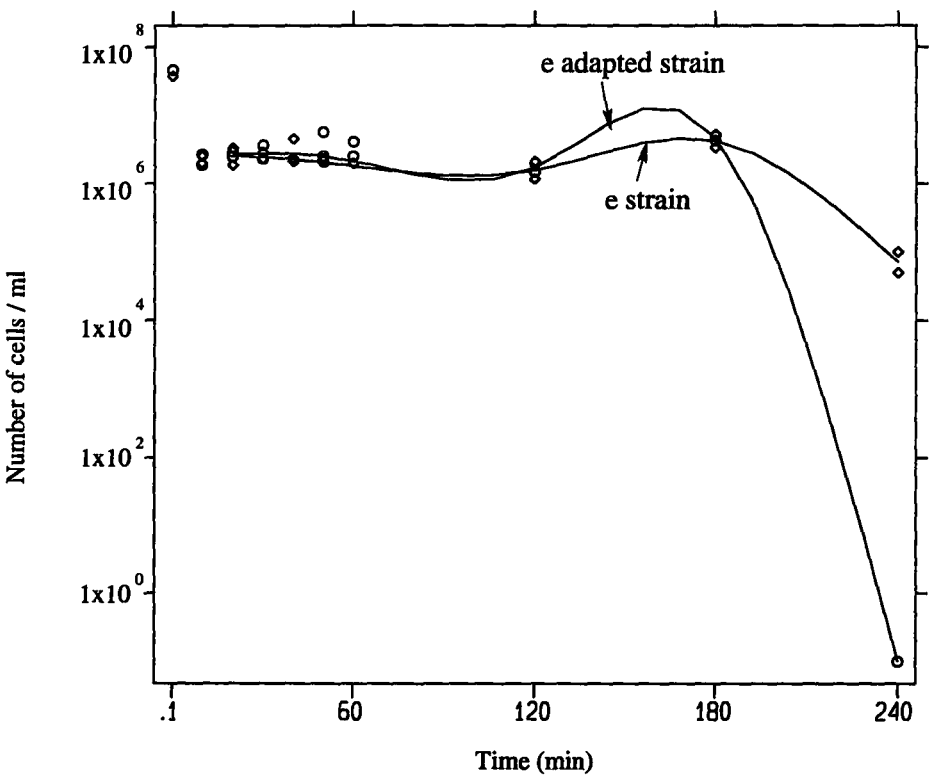
Number of cells / ml
C strain
C adapted strain
.1
60
120
180
240
Time (min)

b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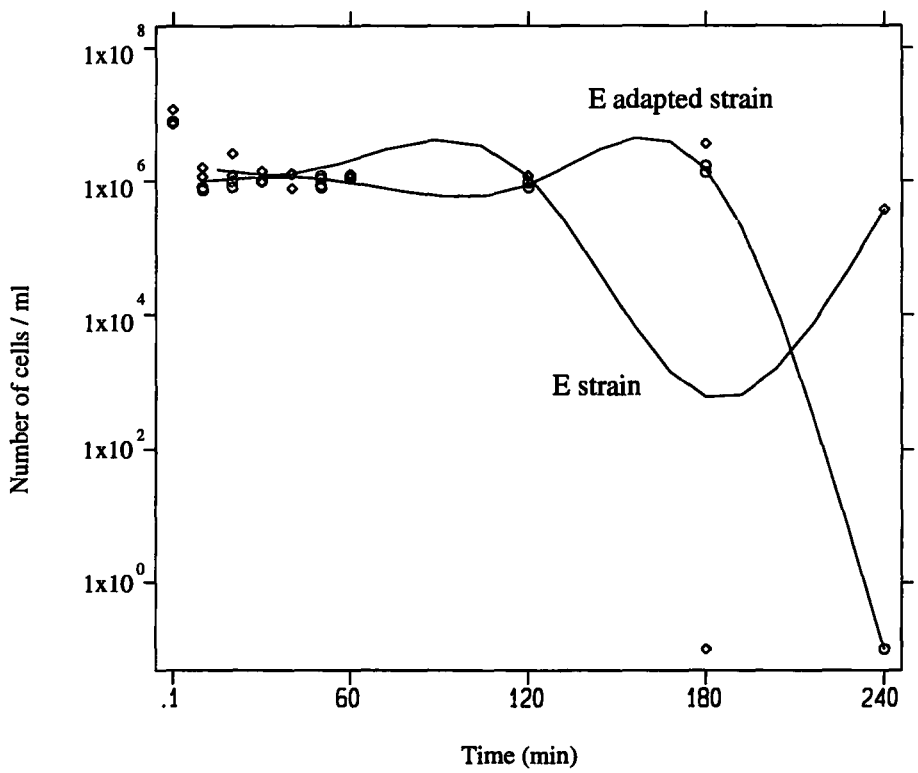
1x10^6
1x10^4
1x10^2
1x10^0
Number of cells / ml
e adapted strain
e strain
.1
60
120
180
240
Time (min)

c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1x10^6
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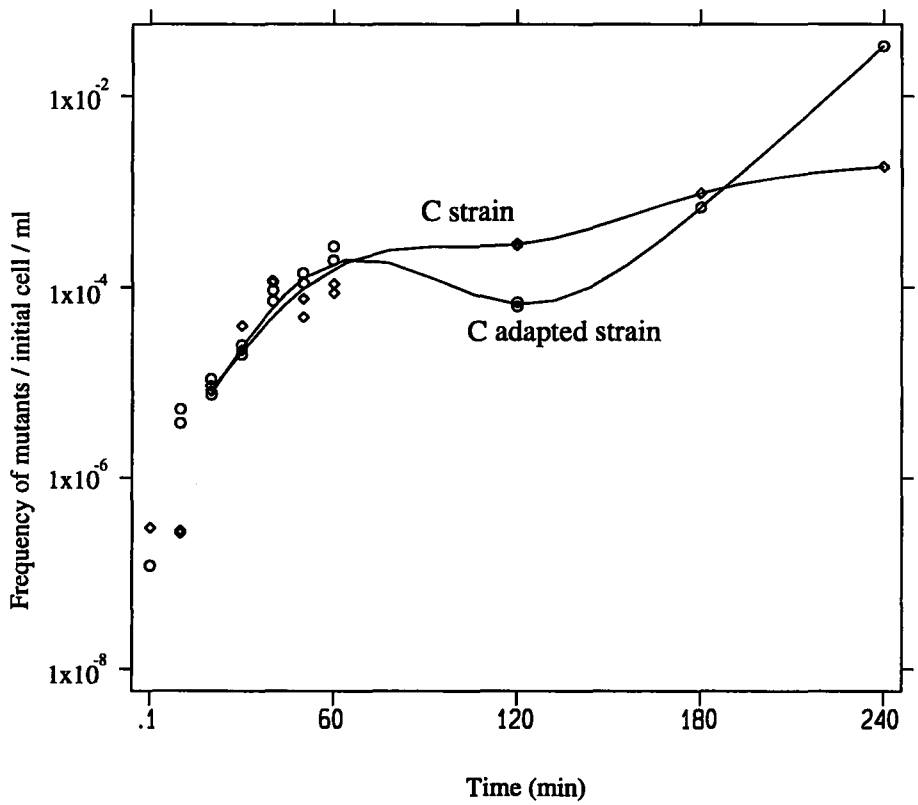
Number of cells / ml
E adapted strain
E strain
.1
60
120
180
240
Time (min)

d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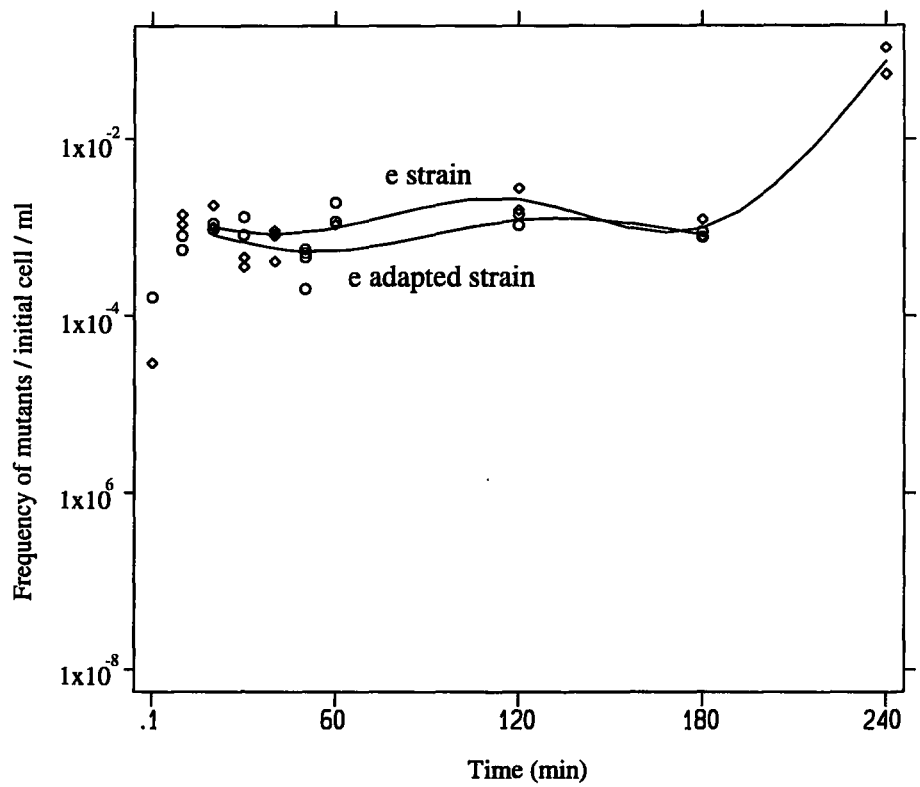
Frequency of mutants / initial cell / ml
C strain
C adapted strain
.1
60
120
180
240
Time (min)

e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e adapted strain
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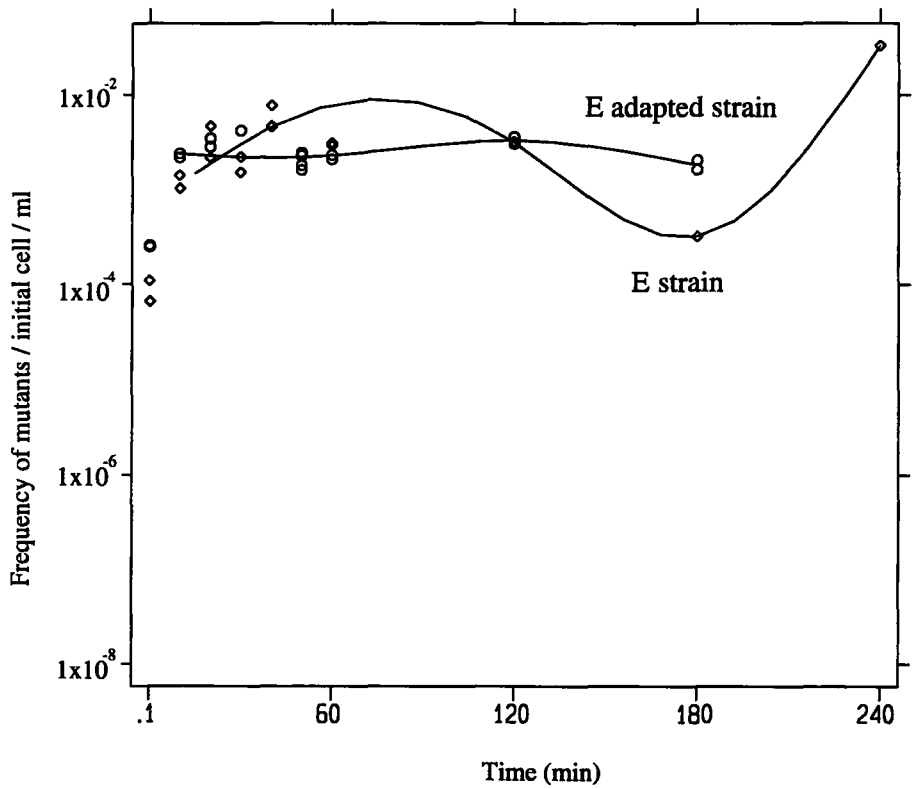
60
120
180
240
Time (min)

f


E adapted strain
E strain
Frequency of mutants / initial cell / ml
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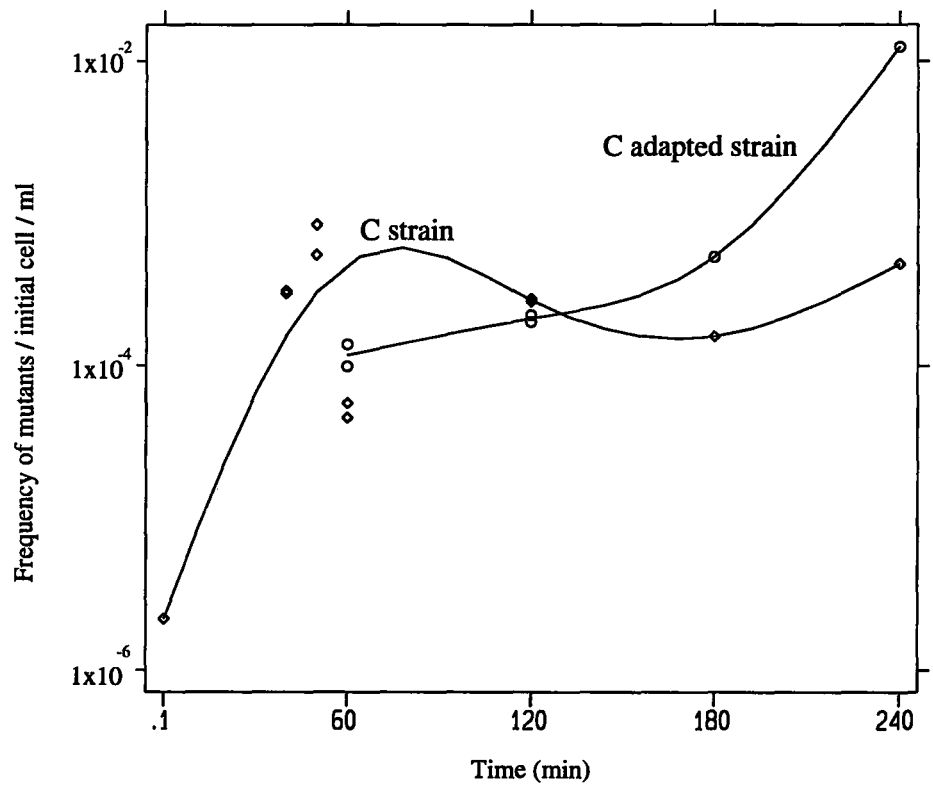
.1
60
120
180
240
Time (min)

g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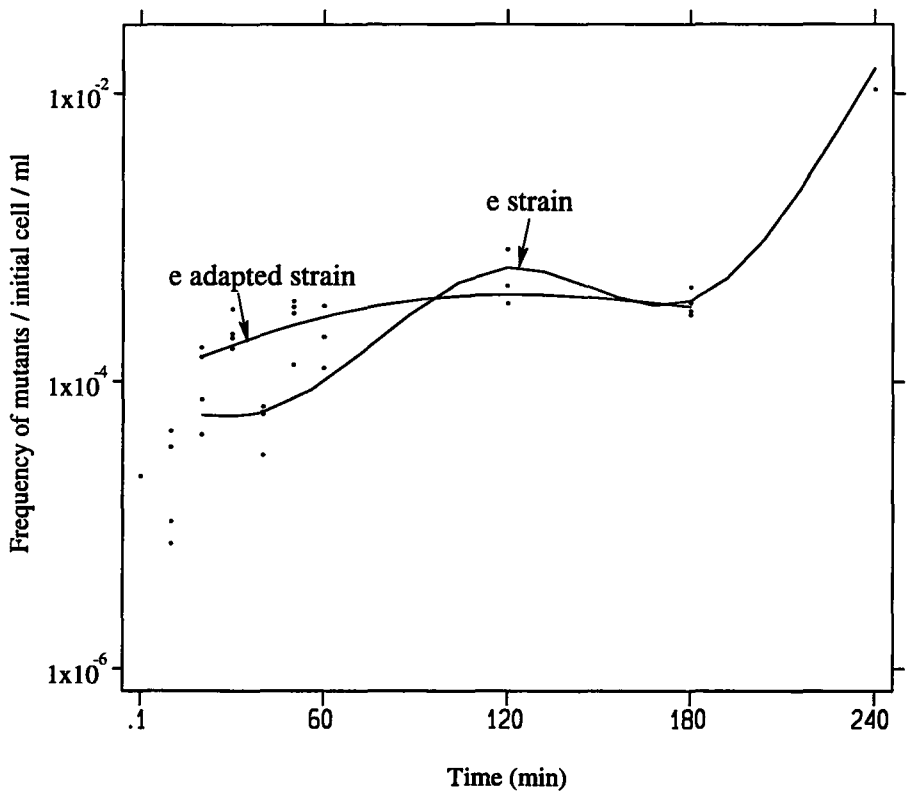
Frequency of mutants / initial cell / ml
C adapted strain
C strain
.1
60
120
180
240
Time (min)

h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Frequency of mutants / initial cell / ml
e strain
e adapted strain
.1
60
120
180
240
Time (min)

i

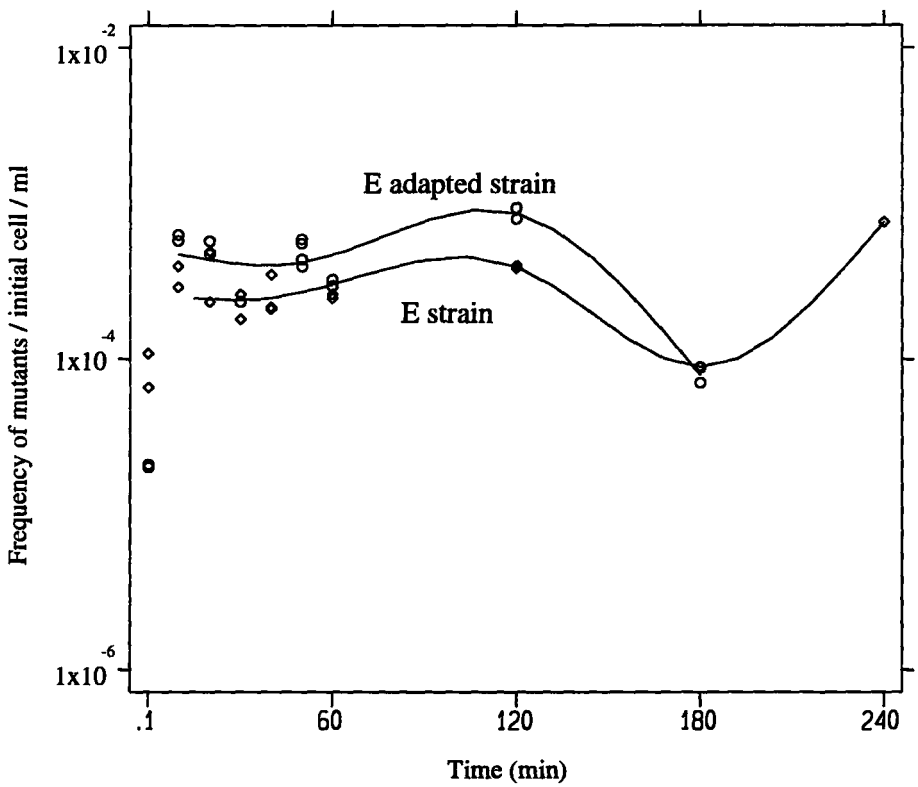
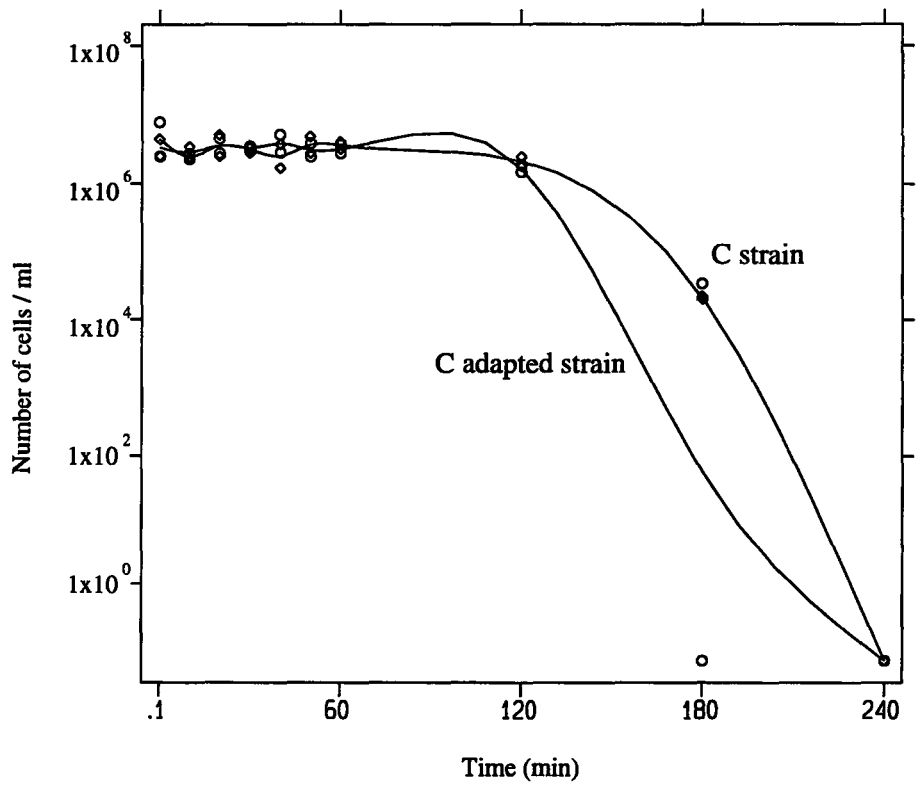

1x10 -2
1x10 -4
1x10 -6
Frequency of mutants / initial cell / ml
E adapted strain
E strain
.1
60
120
180
240
Time (min)

Figure 3.2: The effect of a $100\mu l/10ml$ challenge dose of EMS on survival and mutation frequency of *E. coli* "C" and "C *adapted*" cell lines, "e" and "e *adapted*" cell lines, "E" and "E *adapted*" cell lines. Adapted strains received a $0.2\mu l/10ml$ adaptive dose for 90 min prior to challenge dose. (a-c) survival on LB media, (d-f) mutation frequency on nalidixic plates, (g-i) mutation frequency on rifampicin acid plates. The curves generated are midpoint splines to show general trends in the data and may have greater periodicity than the data warrants.

a


1x10^8
1x10^6
1x10^4
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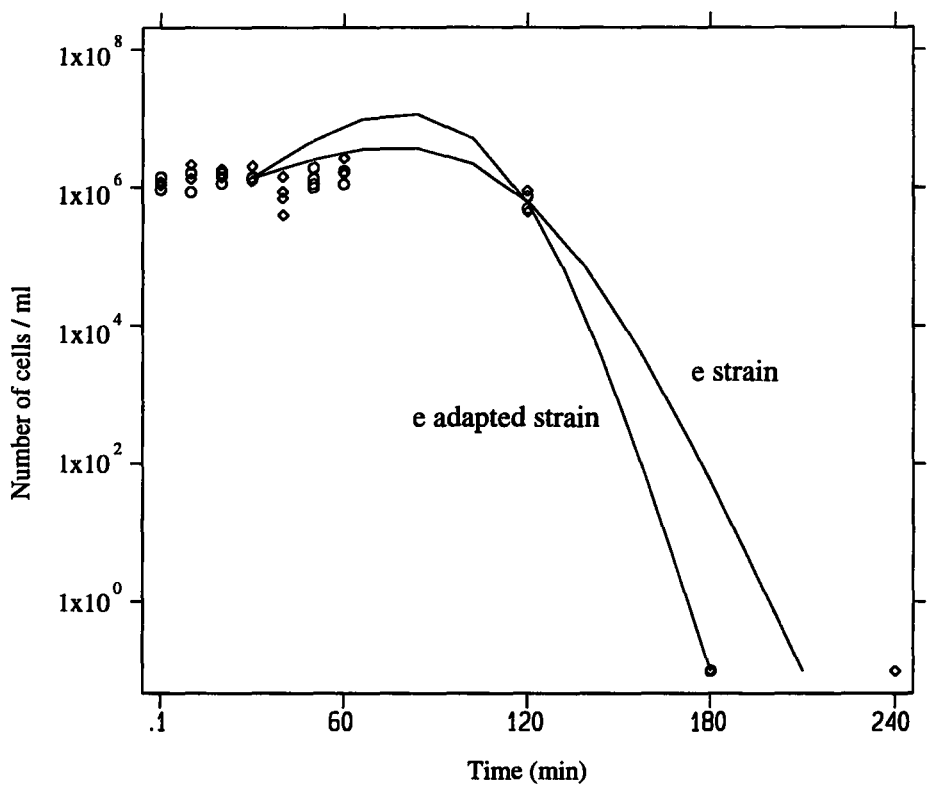
Number of cells / ml
C strain
C adapted strain
.1
60
120
180
240
Time (min)

b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Number of cells / ml
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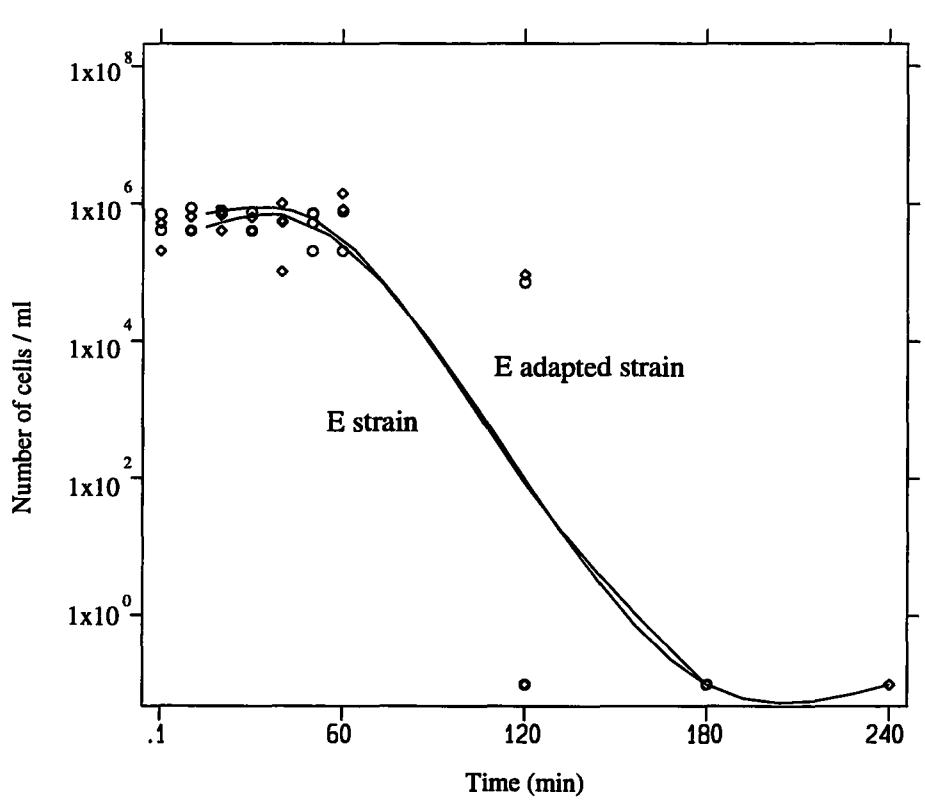
e adapted strain
.1
60
120
180
240
Time (min)

c


1x10^8
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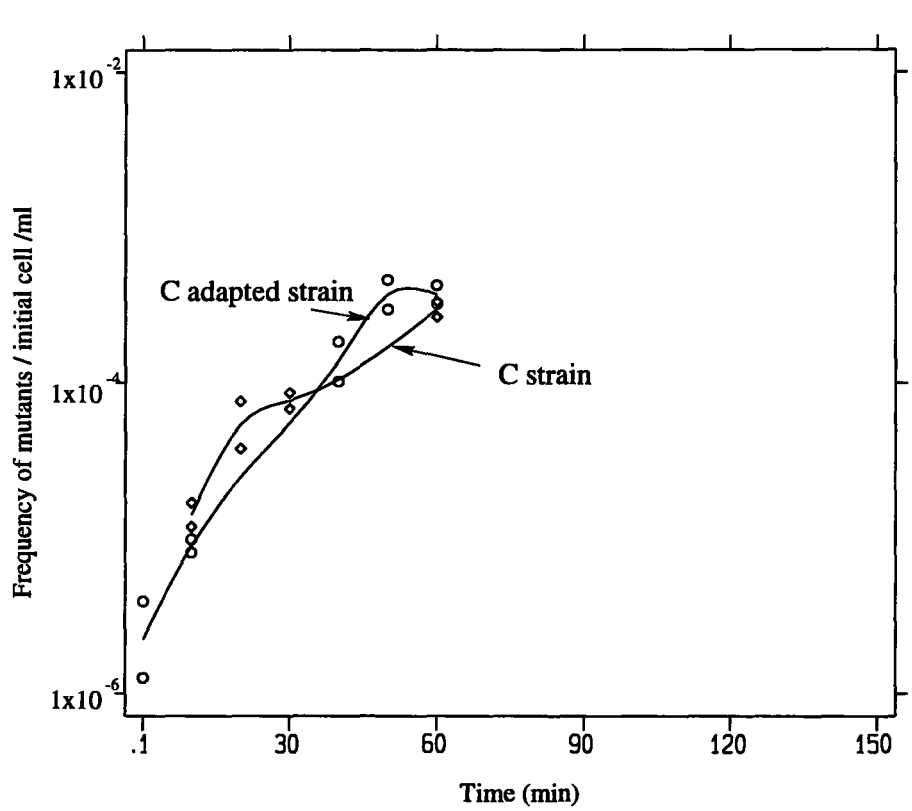
Number of cells / ml
E adapted strain
E strain
.1
60
120
180
240
Time (min)

d


1x10-2
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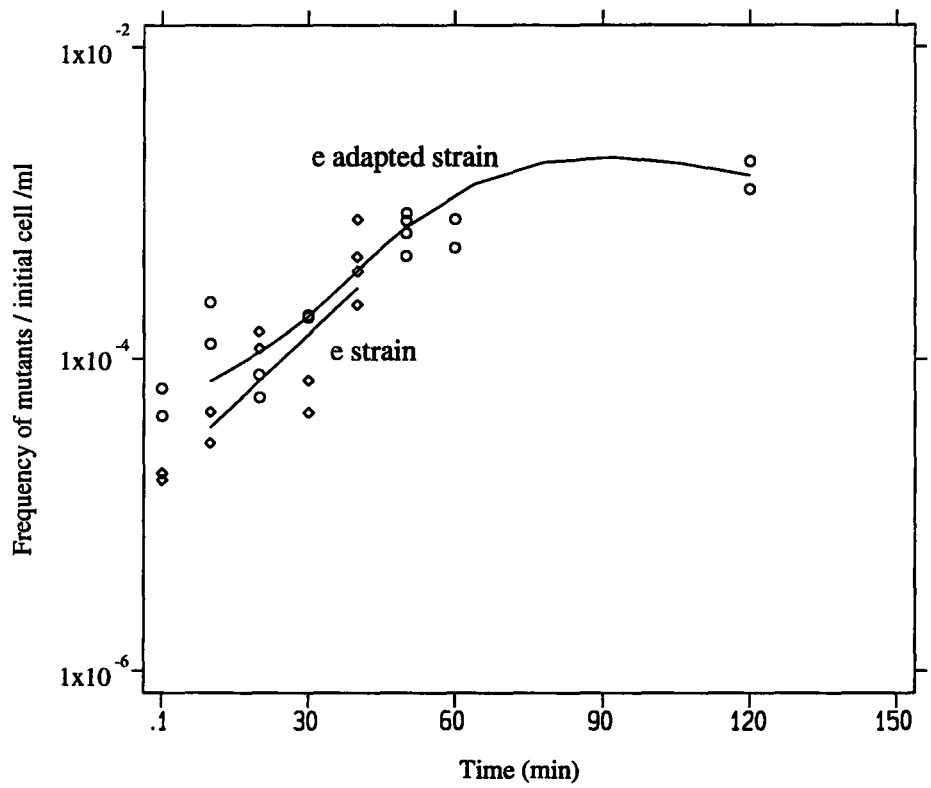
1x10-6
Frequency of mutants / initial cell /ml
C adapted strain
C strain
.1
30
60
90
120
150
Time (min)

e


1x10^-2
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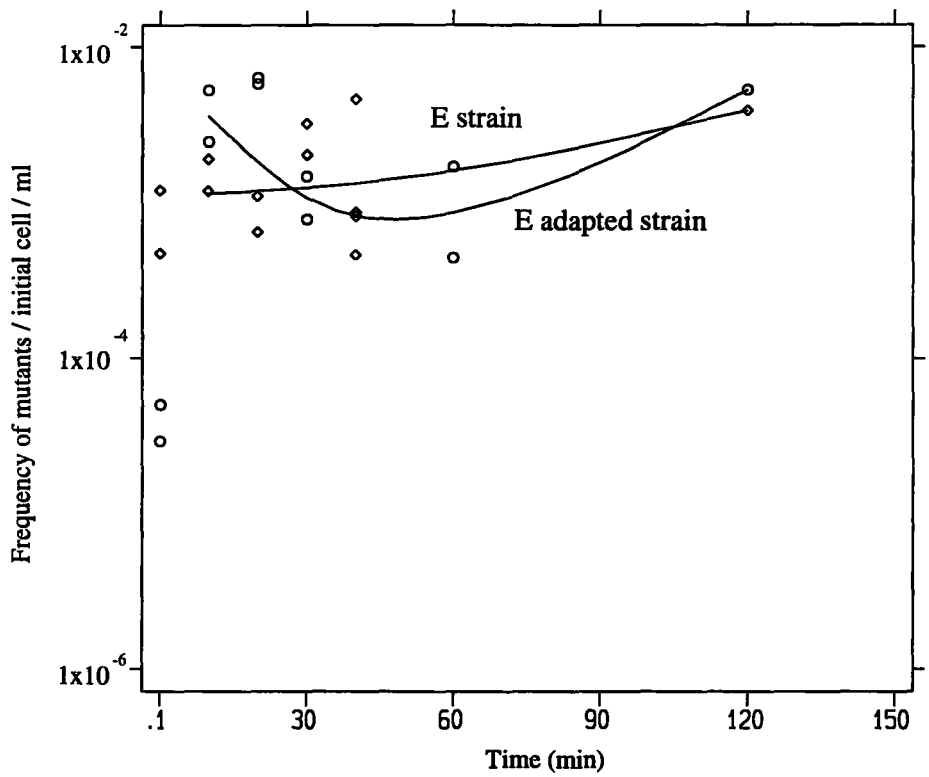
1x10^-6
Frequency of mutants / initial cell /ml
e adapted strain
e strain
.1
30
60
90
120
150
Time (min)

f


1x10^-2
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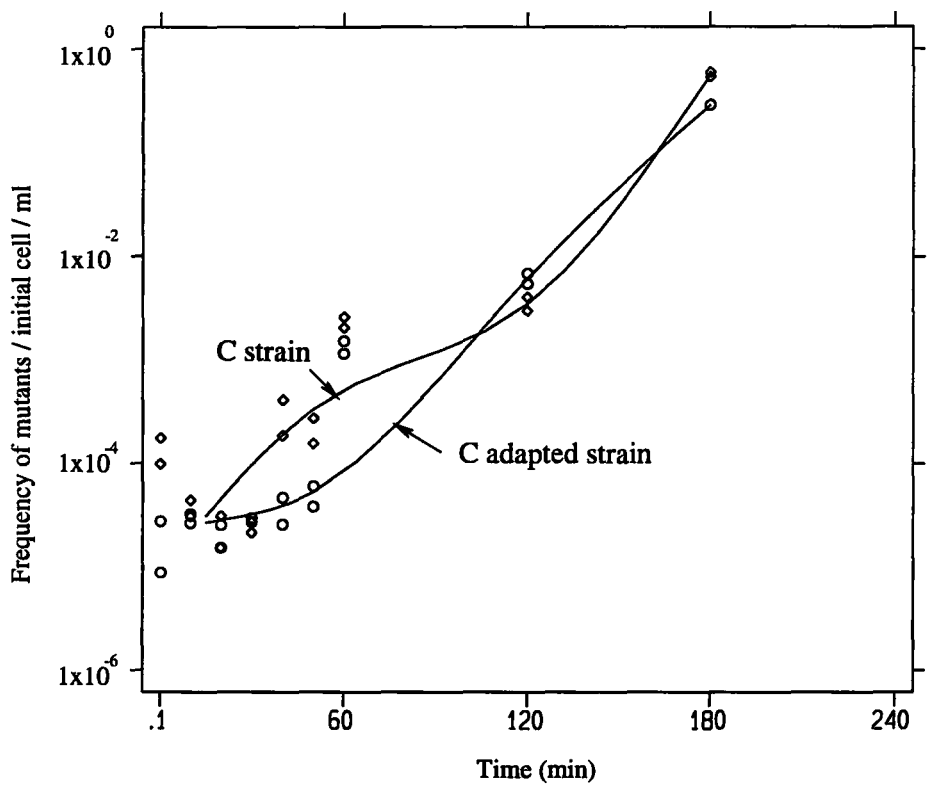
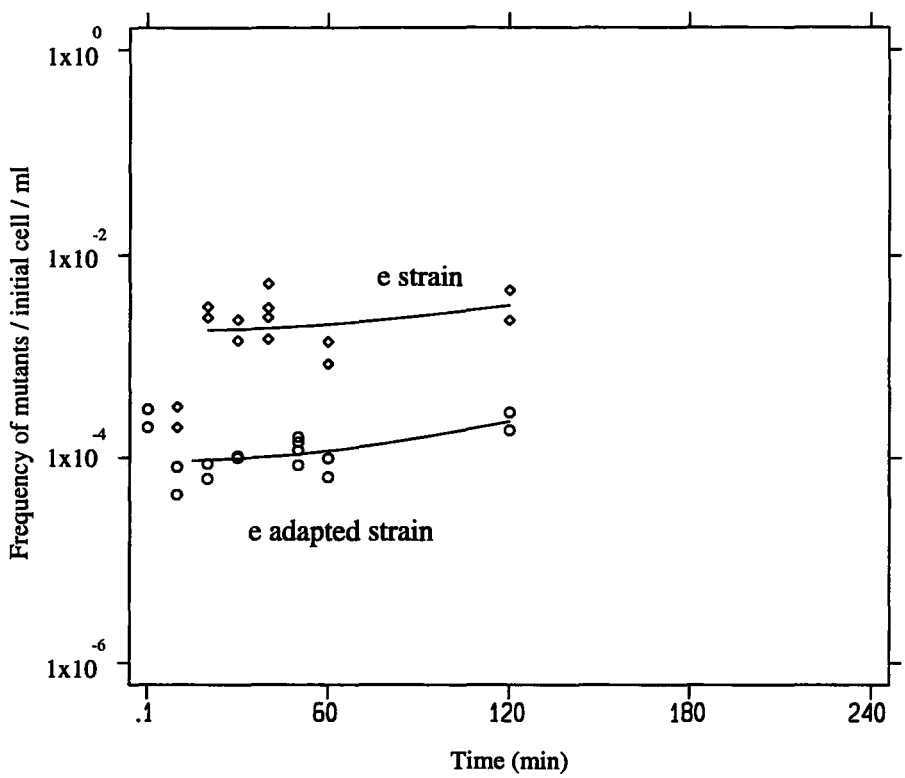
1x10^-6
Frequency of mutants / initial cell / ml
E strain
E adapted strain
.1
30
60
90
120
150
Time (min)

g


Frequency of mutants / initial cell / ml
1x10^0
1x10^-2
1x10^-4
1x10^-6
C strain
C adapted strain
.1
60
120
180
240
Time (min)

h


Frequency of mutants / initial cell / ml
1x10^0
1x10^-2
1x10^-4
1x10^-6
e strain
e adapted strain
.1
60
120
180
240
Time (min)

i

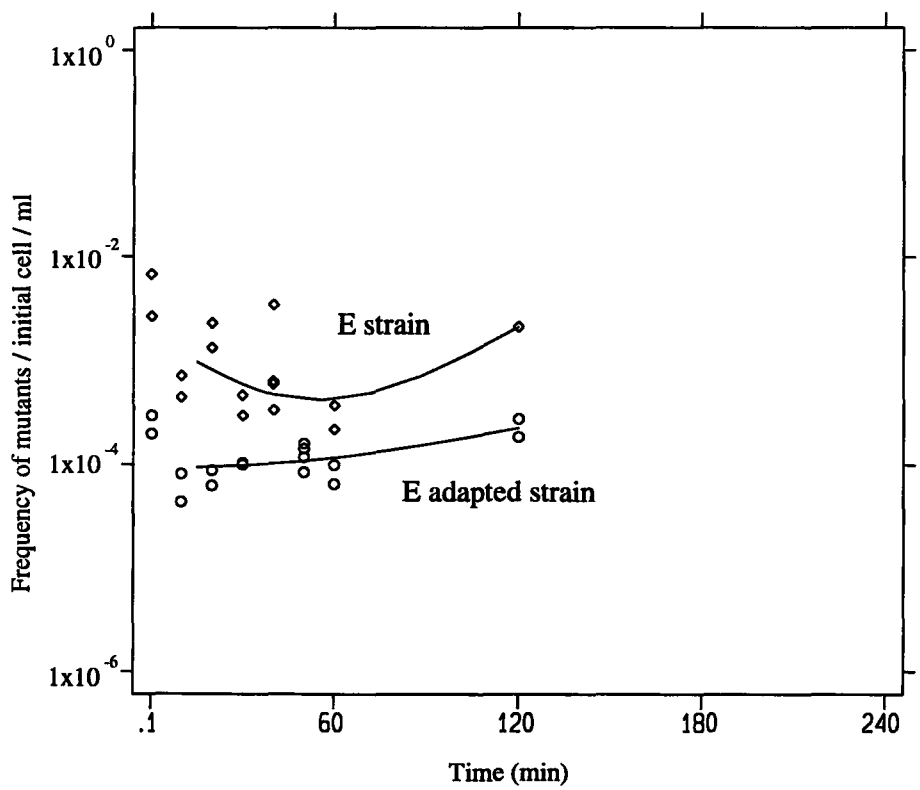

1x10^0
1x10^-2
1x10^-4
1x10^-6
Frequency of mutants / initial cell / ml
E strain
E adapted strain
.1
60
120
180
240
Time (min)

Figure 3.3: The effect of a 100μl/10ml challenge dose of MNNG on survival *E. coli* of "C" and "C *adapted*" cell lines, "e" and "e *adapted*" cell lines, "E" and "E *adapted*" cell lines. The curves generated are midpoint splines to show general trends in the data and may have greater periodicity than the data warrants.

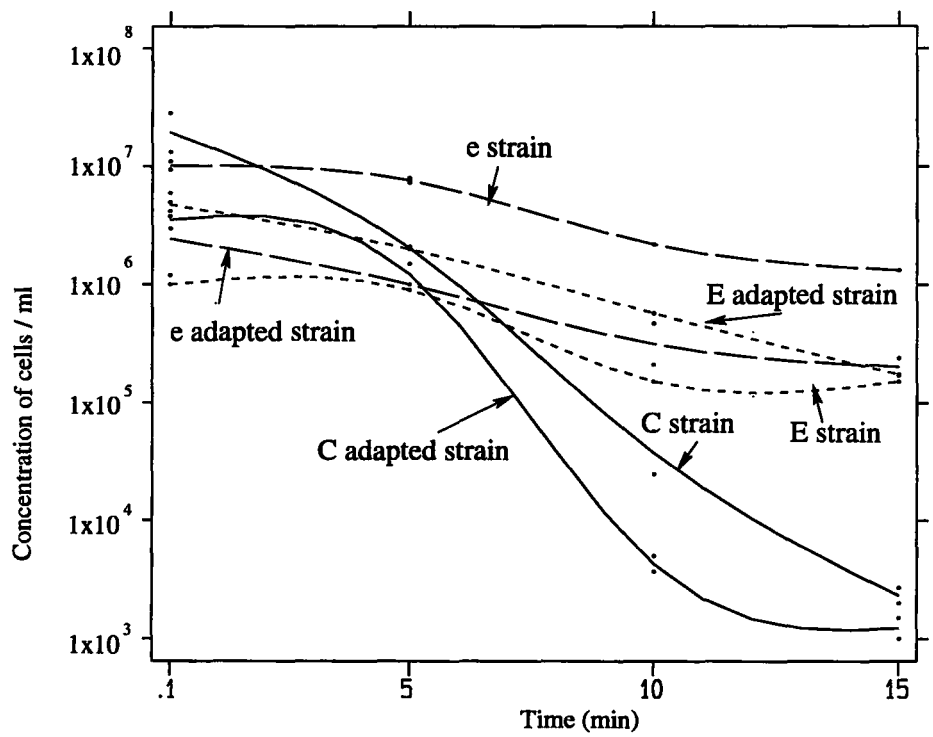
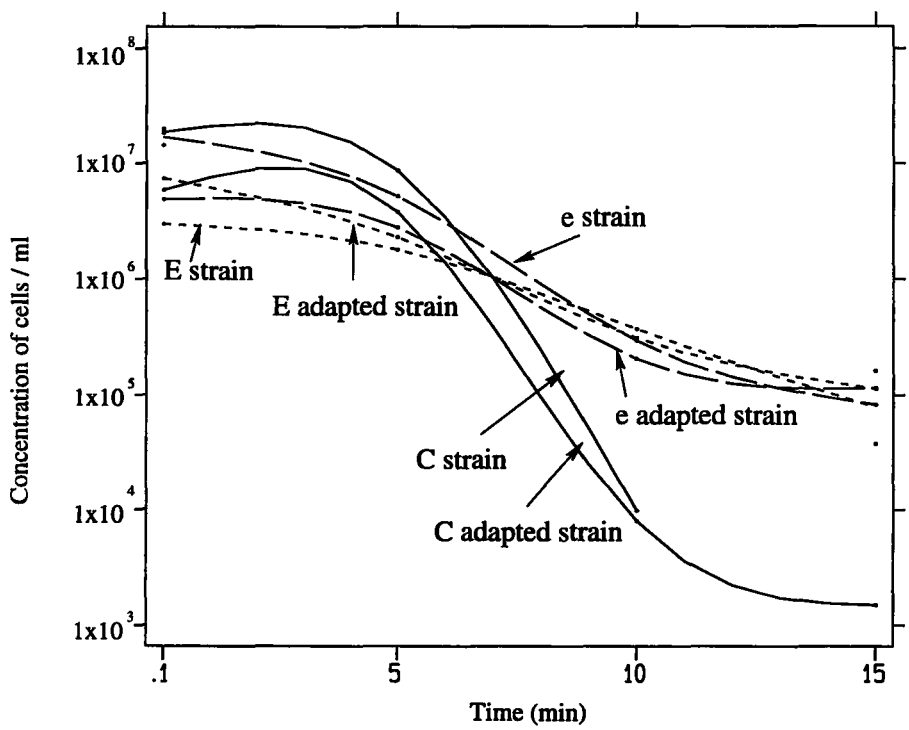

Concentration of cells / ml
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
.1
5
10
15
Time (min)
e strain
E adapted strain
e adapted strain
C strain
E strain
C adapted strain

Table 3.1: Lethal Time (LT_{50}) (min) measurements following a 100μl/10ml challenge dose of MNNG. LT_{50} values were determined by extrapolation from the corresponding survival curves

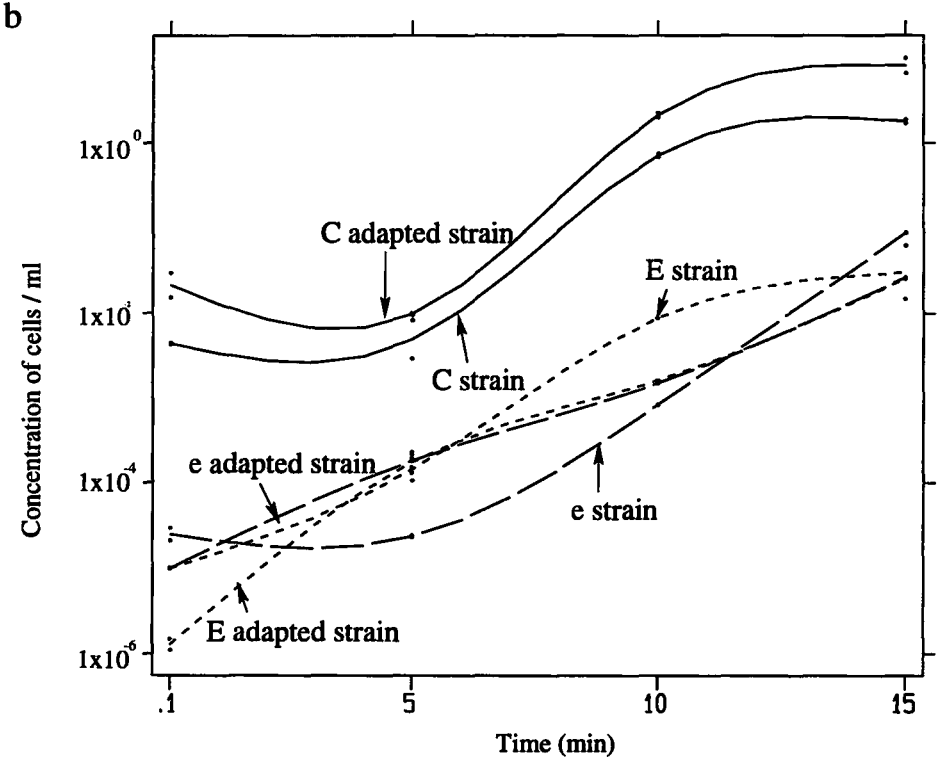
Cell line	LT_{50} (min)
C	5.1
C^A	6.1
e	3.2
e^A	6.3
E	6.3
E^A	4.8

Figure 3.4: The effect of a 100μl/ml challenge dose of MNNG on survival and mutation frequency of *E. coli* "C" and "C ^{adapted}", e and "e ^{adapted}" cell lines, "E" and "E ^{adapted}" cell lines : (a) survival on LB media,(b) mutation frequency on kanamycin plates, (c) mutation frequency on nalidixic acid plates. The curves generated are midpoint splines to show general trends in the data and may have greater periodicity than the data warrants.

a


1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
Concentration of cells / ml
E strain
E adapted strain
e strain
e adapted strain
C strain
C adapted strain
.1
5
10
15
Time (min)

b


Concentration of cells / ml
1x10^0
1x10^-2
1x10^-4
1x10^-6
C adapted strain
C strain
E strain
e adapted strain
e strain
E adapted strain
.1
5
10
15
Time (min)

c

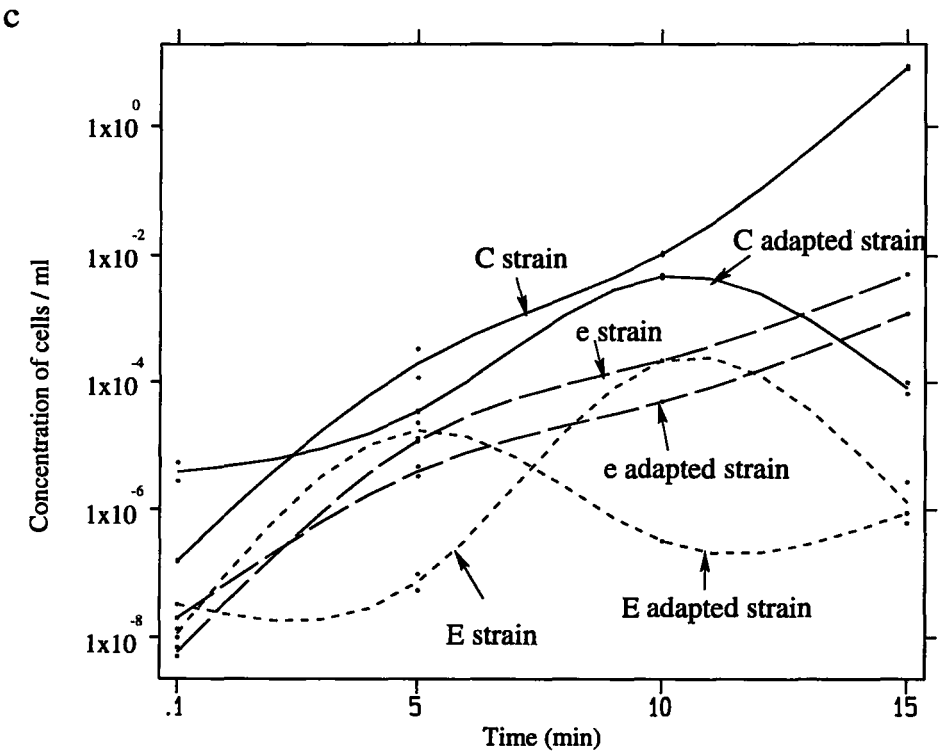

Concentration of cells / ml
1x10^0
1x10^-2
1x10^-4
1x10^-6
1x10^-8
C strain
C adapted strain
e strain
e adapted strain
E strain
E adapted strain
.1
5
10
15
Time (min)

Table 3.2: Lethal Time (LT_{50}) (min) measurements following a 100μl/10ml challenge dose of MNNG. LT_{50} values were determined by extrapolation from the corresponding survival curves

Cell line	LT_{50} (min)
C	1.7
C^A	5.0
e	6.2
e^A	4.2
E	5.5
E^A	6.2

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Chapter 4

Competitive Fitness Estimates

4.1 Introduction

Two of the key elements of biological evolution are the adaptation of organisms to their environment and the divergence of populations and species. Evolutionary biologists endeavour to elucidate the dynamics of these processes and to understand the contributions of natural selection, chance events and historical constraints (Lenski *et al.* 1991). A wide range of evolutionary questions have been addressed using long-term selection experiments with microbial populations (Elena and Lenski 1997; Korona 1996). In general the long-term evolutionary studies conducted in continuous culture devices can be classified into two distinct categories; the study of adaptive evolution and the study of natural selection. The former follows the adaptive evolutionary path of a population in a defined environment, while the latter characterizes

the effects of changes in genetics or environment on selection coefficients (Dykhuizen 1993).

It is suggested that the key factor in the ability of microbial populations to survive diverse environments is their large populations sizes. Bacterial populations often face many environmental growth inhibiting challenges and as a consequence they have evolved survival mechanisms that enable them to cope and prevail against extreme challenges. Since these survival mechanisms are dependent upon the appropriate genetic variables it has been suggested that the organization of these genetic variables may be modulated by biochemical complexes that respond to physiological inputs and up-regulated during times of starvation or other stresses, increasing population fitness and thereby serving an adaptive function (Lenski 1995). These survival mechanisms may be purely incidental, while others may be a consequence of prior long-term evolution that act as a form of insurance implemented at the time of challenge (Koch 1993).

Upon introduction into novel environments replicate populations of *E.coli* show similar fitness trajectories, and undergo initial rapid increases in mean fitness followed by a plateau (Lenski *et al.* 1991; Lenski and Travisano 1994). The deceleration of fitness increases over the course of the experiment is a common feature in long-term evolutionary studies and suggests that intense selection pressure triggers the rapid fitness increase as a result of the initial change in environment (Lenski and Travisano 1994). The leveling off of fitness trajectories may be a consequence of an imposed limit to adaptive evolution due to metabolic constraints despite adaptive mutations directing metabolic activity to faster growth (Korona 1996). Another

suggestion has been a limitation on fitness increase due to a finite number of classes of beneficial mutations that eventually lead to a diminishing rate of increase in mean fitness (Lenski *et al.* 1991). These observations also imply that the evolutionary process may follow more of a predictable path rather than being as unpredictable and chaotic as often suggested by evolutionary biologists (Dykhuizen 1993).

In previous studies long-term adapted cell lines "e" and "E" continually exposed to low doses of the alkylating agent ethyl methane sulfonate (EMS) for over 3000 generations cell lines have demonstrated significantly enhanced resistance to the lethal effects and a higher tolerance to the mutagenic effects of large challenge doses of EMS relative to the long-term non-adapted control cell lines "C" (section 2). Competition experiments are most commonly used to determine direct estimates of fitness of derived populations relative to their common ancestor in both selective and novel environments. Competition experiments were conducted between the adapted "E"/"e" cell lines against the control "C" cell lines in order to measure the relative fitness of the competitors over several generations and to determine the fitness response of the cell lines both in the absence and in the presence of EMS treatment.

4.2 Methods

Bacterial Lines. The experiments were conducted using cell lines from an ancestral *E. coli* CSH100 *ara* Δ (*gpt-lac*)5 mating type F′ *lacpro* A^+ B^+]clone obtained from Cold Spring Harbour Laboratory. This strain was separated into six individual lines that were then maintained by serial culture as pure lines for over 10 months. At

this point in time these ancestral cell lines ("generation 0") were further propagated for an additional 24 months (over 4000 generations) for long-term adaptation experimental analysis. The clones use for the following experiments had been cultured for approximately 1 year (generation 1600).

Culture Conditions. Three replicates of two experimental treatment lines were established and have been maintained for approximately 3 years at 37^0C. All lines were propagated with M9 minimal medium and maintained daily by 1:100 serial dilution into 10 ml of fresh medium. The EMS adapted lines designated as "E" were treated daily with 25μl of EMS following serial dilution. The control lines designated as "C" were grown in the absence of EMS. All other conditions were identical for both the "E" and the "C" lines. In most of the experiments conducted a third cell line was established using an inoculate from the "E" EMS adapted line. This line designated "e" was grown up overnight (approximately 8 generations) in the absence of EMS in order to eliminate any physiological changes and distinguish between phenotypic acclimation and genetic modification.

Chemical Reagents. Ethyl methane sulfonate (EMS), agar and all necessary supplements were obtained from Sigma Chemical Co.

Bacterial Growth Medium. Liquid growth media consisted of minimal M9 solution supplemented with 20% glucose, 1M $MgSO_4$, thiamine and 0.5% thiamine per litre.

The founding lines used in this experiment possessed one of two arabinose-utilization marker states in order to identify the lines in the fitness assays. Lines were initially

tested against themselves to eliminate any differences in fitness due to arabinose marker state. The marker state was also utilized to "monitor" any cross contamination. The stability of the arabinose marker state was tested in both control and EMS treated experimental conditions of all the lines. Cells were plated on MacConkey indicator plates each day for a total of three days, and colonies were scored for colour. Competition experiments were conducted in culture conditions containing an EMS treatment as well as control culture conditions. Both competitors were pre-incubated overnight in the experimental culture conditions in order to acclimatize both lines physiologically to the culture conditions. Adapted cultures pre-incubated in EMS were designated as "E" lines while adapted cultures pre-incubated in the absence of EMS were designated as the "e" lines. A single colony isolate was grown overnight and a volume of $50\mu l$ containing a concentration of approximately 1×10^9cells/ml of each competitor was added to 10ml of fresh M9 medium. Samples were taken in order to determine the initial densities of the competing lines. These samples were plated on MacConkey arabinose medium to distinguish the competitors on the basis of the arabinose-utilization marker state. The mixed cultures were maintained at 37^0C and final samples were taken at following 24h of competition in order to determine the final densities of the EMS adapted and control lines. The relative fitness (W) was measured as the ratio of the number of doublings for the EMS adapted "E" and the control "C" cell lines (Travisano 1997). A value of 1 indicates that the two clones have equal fitness; a value greater than 1 indicates that the cell line carrying the ara^+ marker has adapted evolutionarily: while a value less than 1 indicates that the clone carrying the ara^- marker has adapted evolutionarily. Three replicate competition experiments were conducted for each cell line-treatment and arabinose marker state

competition. Each replicate fitness estimate is treated as three independently equally accurate measurements.

4.3 Results

The major results of the competition experiments and the relative fitness estimates are given in Table 4.1 and 4.2 respectively. Table 4.1 indicates the proportion of ara^+ vs. ara^- cells in the initial sample (day 0) and the final sample (day 1) following 18 hours of competition. Table 4.2 indicates the relative fitness estimates calculated as the ratio of the number of doublings for the EMS adapted and the control cell lines. A value of 1 indicates that the two clones have equal fitness; a value greater than 1 indicates that the cell line carrying the ara^+ marker has adapted evolutionarily; a value less than 1 indicates that the clone carrying the ara^- marker has adapted evolutionarily. The relative fitness values for all of the compeition assays were summarized into a bar graph (Figure 4.1). The bar indicates the measure of the median relative fitness of the "C" control cell lines relative to the "E"/"e" adapted cell lines. A value of 1 indicates that the two clones have equal fitness; a value greater than 1 indicates that the "C" control cell line has adapted evolutionarily: while a value less than 1 indicates that "E" "e" cell line has adapted evolutionarily. Figure 4.1 indicates that the "E"/"e" cell lines have a higher relative fitness than the "C" control cell lines in both the presence and in the absence of EMS treatment. Due to the high degree of variabilty among the individual relative fitness estimates the median fitness estimate for each treatment is not an accurate indication of the level of evolutionary adaptation to the EMS treated

environment and therefore these median estimates are not comparable.

The stability of the arabinose marker state was tested in both control and EMS treated experimental conditions for all of the cell lines. LB cultures inoculated with cell lines carrying only one arabinose marker state were sampled and plated daily for three days in order to monitor for arabinose marker state reversions. The arabinose marker appeared stable for all of the cell lines in both the presence and in the absence of EMS treatment.

Self competition experiments were carried out in identical cell lines differing only in their arabinose marker state were placed into competition in order to determine if the arabinose marker state was selectively neutral in both the presence and the absence of EMS treatment (Table 4.1 and 4.2). Comparison of the mean derived fitness estimates indicates that only 2 out of the 16 self competition experiments (e1a^{+}vs e1a^{-}) and (C2b^{+} vs C2b^{-}) had fitness estimates indicating equal relative fitness between the two competing clones. Although these observations would initially suggest that the arabinose marker state may not be neutral in our experimental conditions, the fitness estimates from the self competition experiments do not indicate a clear fitness advantage or disadvantage dependent upon ara marker state and the fitness advantages appear to vary with clone treatment and arabinose marker state. Standard deviation values indicate that there is significant variability among the three replicate estimates and therefore it is not possible to determine if the arabinose marker is selectively neutral or if it confers a fitness advantage to the cell lines.

The relative fitness estimates from the competition experiments conducted in EMS treated environments suggest that the EMS "E" adapted cell lines resulted in a

higher relative fitness compared to the "C" control cell line. (Table 4.2). This fitness advantage varied from a slight advantage as seen in E2b+ vs C2b- (mean fitness ratio 1.052) to a significant advantage as seen with E2a+ vs C2a- (mean fitness ratio 4.287). These results suggest that the "E" cell lines have adapted evolutionarily to an EMS treated environment, however there was significant variability observed in all of the competition experiments and therefore based on these observations we cannot conclude a significant adaptive advantage for the "E"/"e" adapted cell lines.

In competition experiments conducted in untreated cultures, 7/9 competition experiments yielded fitness ratios suggesting the "E" cell line to have a fitness advantage over the "C" cell lines (Table 3.2). As seen in the EMS treated environment, the fitness advantages varied from a slight advantage as seen in e2a+ vs C2a- (fitness ratio 1.017) to a significant advantage seen in e3b+ vs c2b- (fitness ratio 4.43). These results suggests that the "E" EMS adapted cell lines have a higher overall relative fitness compared to the "C" control lines in the untreated experimental environments, however due to the significant variability observed in 8 out of the 9 competition fitness estimates the competition results are inconclusive.

4.4 Discussion

Numerous published papers have shown the arabinose marker to be selectively neutral in minimal glucose medium and therefore this marker has been predominately used for bacterial competition assays to get direct estimates of fitness (Elena and Lenski 1997; Bennett, Dao and Lenski 1990; Bennett and Lenski 1996; Sniegowski,

Gerrish and Lenski 1997). The relative fitness estimates calculated from the self competition experiments indicated that the majority of the clones founded from the same culture differing only in their arabinose marker states do not have equal fitnesses. These results however, do not necessarily indicate that the arabinose marker state is not neutral, since the fitness estimates do not show a clear fitness advantage or disadvantage dependent upon ara marker state. The fitness advantages observed in the self competition experiments appear highly variable and change with treatment and cell line. This initially suggested that the clones themselves differ slightly in their relative fitness independent of arabinose marker state. Studies have shown that replicate bacterial populations evolving in identical experimental environments can diverge significantly from one another in mean fitness as a result of the populations approaching varying fitness peaks in the adaptive landscape (Lenski and Travisano 1994). Despite the significant between population genetic variance in mean fitness often detected in replicate populations, significant within population variance in not usually observed (Lenski *et al.* 1991; Lenski and Travisano 1994). Therefore it is unlikely that the extreme fitness differences are due to genetic variance between clones, or a result of the arabinose marker state. These results are most likely a consequence of inaccurate fitness estimates due to a difficulty in discriminating between ara^+ and ara^- clones on MacConkey indicator media. One of the problems often encountered with the MacConkey media are diminished colour reactions when plates are too crowded with colonies (Miller 1992). Since two dilutions were made of each culture sample plated, diminished colour reactions may have affected the plates containing a higher dilution of cells therefore skewing the cell counts. Studies using arabinose marker states in order to determine direct fitness estimates of competing

strains of bacteria in culture use terazolium arabinose (TA) indicator agar instead of MacConkey media in order to accurately distinguish between ara^+ and ara^- colonies (Bennett, Dao and Lenski 1990; Bennett and Lenski 1996; Sniegowski, Gerrish and Lenski 1997). However, preliminary testing of the MacConkey and tetrazolium arabinose agar indicated no significant differences in ability to distinguish ara^+ and ara^- bacterial colonies. Due to the expense of tetrazolium media and the large scale of the competition experiments, MacConkey agar was chosen as the indicator media.

The derived fitness values from the competition experiments suggest that the "E" EMS adapted clones have an overall higher relative fitness compared to the "C" control clones in both the EMS treated and the untreated experimental culture conditions. These results would be expected for competition in EMS treated media since previous survival and mutation frequency data on these long-term evolved bacterial populations (described in detail in section 2) indicated a higher level of resistance to the mutagenic and lethal effects of EMS treatment compared to "C" control cell lines suggesting a higher relative fitness.

The results for the competition experiments conducted in untreated culture conditions were unexpected since bacteria adapted to a particular environment should be significantly more fit than bacteria adapted to other environmental conditions. Therefore "C" control line was expected to out-compete the "E" cell line in the untreated culture conditions. In retrospect the suggested high relative fitness of the "E" clones is not that surprising. Environment specific adaptation may also result in improved fitness in novel environments (Travisano 1997). Studies have indicated that populations adapting to specific thermal regimes do not necessarily compromise

fitness in other thermal regimes and may actually lead to fitness advantages to numerous novel environments. Competitive fitness assays conducted between bacteria acclimated to specific temperatures indicated that bacteria acclimated to 32^0C were more fit than bacteria acclimated at 41.5^0C in both 32^0C and 41.5^0C experimental culture conditions (Leroi, Bennett and Lenski 1994). In experimental conditions containing unlimited resources, bacterial adaptation tends to be unidirectional towards a higher growth rate (Korona 1996) and therefore a higher relative fitness in competition experiments is usually the result of a stronger performance in one or more of the phases of population growth dynamics (Leroi, Bennett and Lenski 1994). Therefore it is quite possible that the adaptive traits selected for in the "E" EMS adapted cell lines also improves the growth rate of these cells in untreated cultures.

Despite these promising findings the reliability of the fitness estimates from this analysis is questionable. The self competition experiments clearly indicate that identical strains containing opposite arabinose marker states were not balanced with regards to fitness. The significantly high variability among replicate fitness estimates ara^+ and ara^- colonies possibly due to diminished colour reactions. As a result no conclusions regarding adaptive advantages can be drawn from our observations. This problem may be resolved by using a more accurately discriminating indicator media such as tetrazolium arabinose agar. In retrospect, the self competition experiments should have been conducted prior to, and not simultaneously with the other direct "E" vs "C" competition experiment. This would have enabled the experiments to be fully balanced with respect to marker states (Travisano 1997). Although the results from this analysis remain inconclusive, these preliminary experimental find-

ings suggest an enhanced fitness response in the "E" EMS adapted cell lines to EMS treated conditions and it would be worth while to replicate the above experiments incorporating the suggested experimental modifications.

Table 4.1: Competition fitness assay colony counts indicating the proportion of ara^+ vs. ara^- colonies on day 0 and day 1. The entries with 0 were experiments where no ara^+ cells were found and hence the precise fitness value can not be calculated. Entries with "ND" indicate that the corresponding experiment was not carried out. Values in parentheses indicate the total cell count for the corresponding experiment

Strains	EMS	Proportion of ara^+ vs. ara^-						Time
C^+_{1a}/e^-_{1a}	-	0.057	(110)	0.061	(124)	0.081	(122)	0
		0.567	(223)	0.692	(27)	0.627	(490)	1
C^+_{1a}/E^-_{1a}	+	0.620	(40)	0.619	(145)	0.716	(113)	0
		0.144	(135)	0.524	(97)	0.638	(150)	1
e^+_{1a}/C^-_{1a}	-	0.262	(496)	0.261	(534)	0.305	(371)	0
		0.352	(234)	0.344	(101)	0.399	(596)	1
E^+_{1a}/C^-_{1a}	+	0.252	(104)	0.232	(41)	0.266	(438)	0
		0.830	(296)	0.498	(364)	0.307	(238)	1
C^+_{1a}/C^-_{1a}	-	0.506	(218)	0.010	(277)	0.013	(205)	0
		0.155	(309)	0.045	(296)	0.147	(68)	1
C^+_{1a}/C^-_{1a}	+	0.151	(330)	0.109	(280)	0.206	(409)	0
		0.009	(463)	0.031	(268)	0.057	(282)	1
e^+_{1a}/e^-_{1a}	-	0.685	(105)	0.661	(89)	0.721	(292)	0
		0.749	(175)	0.771	(176)	ND	-	1
E^+_{1a}/E^-_{1a}	+	0.802	(224)	0.760	(191)	0.741	(252)	0
		0.486	(192)	0.165	(111)	0.418	(152)	1
C^+_{1b}/e^-_{1b}	-	0.731	(69)	0797	(120)	0.598	(73)	0
		0.736	(132)	0.982	(127)	0.912	(66)	1
C^+_{1b}/E^-_{1b}	+	0.896	(67)	0.810	(93)	0.676	(95)	0
		0.560	(89)	0.610	(50)	ND	-	1
e^+_{1b}/C^-_{1b}	-	0.511	(408)	0.260	(453)	0.245	(458)	0
		0.293	(87)	0.508	(536)	0.566	(419)	1
E^+_{1b}/C^-_{1b}	+	0.663	(216)	0.829	(413)	0.865	(379)	0
		0.957	(236)	0.867	(273)	0.852	(227)	1

Strains	EMS	Proportion of ara^+ vs. ara^-						Time
C^+_{1b}/C^-_{1b}	-	0.058	(300)	0.029	(361)	0.017	(440)	0
		0.131	(418)	0.090	(411)	0.064	(374)	1
C^+_{1b}/C^-_{1b}	+	0.044	(297)	0.066	(255)	0.066	(290)	0
		0.062	(66)	0.067	(349)	0.080	(305)	1
e^+_{1b}/e^-_{1b}	-	0.404	(152)	0.583	(182)	0.419	(220)	0
		0.783	(55)	0.725	(82)	0.858	(242)	1
E^+_{1b}/E^-_{1b}	+	0.822	(240)	0.742	(151)	0.716	(237)	0
		0.455	(11)	0.283	(108)	0.415	(94)	1
C^+_{2a}/e^-_{2a}	-	0.307	(250)	0.213	(322)	0.299	(285)	0
		0.0	(603)	0.0	(36)	0.0	(85)	1
C^+_{2a}/E^-_{2a}	+	ND	-	ND	-	ND	-	0
		0.897	(212)	0.869	(222)	0.974	(218)	1
e^+_{2a}/C^-_{2a}	-	0.563	(160)	0.523	(78)	0.772	(113)	0
		0.709	(116)	0.422	(64)	0.760	(487)	1
E^+_{2a}/C^-_{2a}	+	0.326	(42)	0.231	(133)	0.089	(74)	0
		0.170	(214)	0.284	(46)	0.989	(175)	1
C^+_{2a}/C^-_{2a}	-	0.902	(238)	0.950	(306)	0.904	(321)	0
		0.165	(71)	0.366	(54)	0.222	(88)	1
C^+_{2a}/C^-_{2a}	+	0.711	(143)	0.750	(294)	0.810	(303)	0
		0.958	(143)	0.944	(53)	0.488	(83)	1
e^+_{2a}/e^-_{2a}	-	0.717	(216)	0.333	(6)	0.655	(201)	0
		0.461	(115)	0.475	(132)	0.379	(118)	1
E^+_{2a}/E^-_{2a}	+	0.598	(162)	0.719	(164)	0.688	(155)	0
		0.973	(232)	0.886	(125)	0.947	(107)	1

Strains	EMS	Proportion of ara^+ vs. ara^-						Time
C^+_{2b}/e^-_{2b}	-	0.176	(126)	0.117	(195)	0.073	(380)	0
		0.0	(404)	0.004	(204)	0.003	(260)	1
C^+_{2b}/E^-_{2b}	+	0.234	(243)	0.419	(117)	ND	-	0
		0.0	(238)	0.0	(288)	0.0	(170)	1
e^+_{2b}/C^-_{2b}	-	0.284	(160)	0.118	(301)	0.296	(254)	0
		0.662	(295)	0.562	(294)	0.508	(199)	1
E^+_{2b}/C^-_{2b}	+	0.488	(123)	0.381	(211)	0.334	(491)	0
		0.424	(243)	0.501	(94)	0.325	(300)	1
C^+_{2b}/C^-_{2b}	-	0.667	(298)	0.436	(276)	0.766	(309)	0
		0.552	(155)	0.034	(220)	0.0	(198)	1
C^+_{2b}/C^-_{2b}	+	0.507	(43)	0.823	(96)	0.840	(80)	0
		0.411	(72)	0.996	(158)	0.976	(68)	1
e^+_{2b}/e^-_{2b}	-	0.814	(161)	0.648	(161)	0.687	(150)	0
		0.145	(81)	0.699	(89)	0.257	(85)	1
E^+_{2b}/E^-_{2b}	+	0.293	(166)	0.762	(103)	0.629	(115)	0
		0.997	(219)	0.899	(131)	0.965	(93)	1
C^+_{2a}/e^-_{3a}	-	0.573	(253)	0.616	(139)	0.391	(290)	0
		0.117	(284)	0.626	(265)	0.096	(278)	1
C^+_{2a}/E^-_{3a}	+	0.655	(148)	0.768	(147)	0.529	(176)	0
		0.723	(47)	0.629	(314)	0.713	(87)	1
e^+_{3a}/C^-_{2a}	-	0.063	(271)	0.259	(432)	0.211	(222)	0
		0.581	(569)	0.360	(139)	0.719	(189)	1
E^+_{3a}/C^-_{2a}	+	ND	-	0.295	(26)	0.120	(66)	0
		1.0	(191)	0.984	(159)	0.932	(234)	1

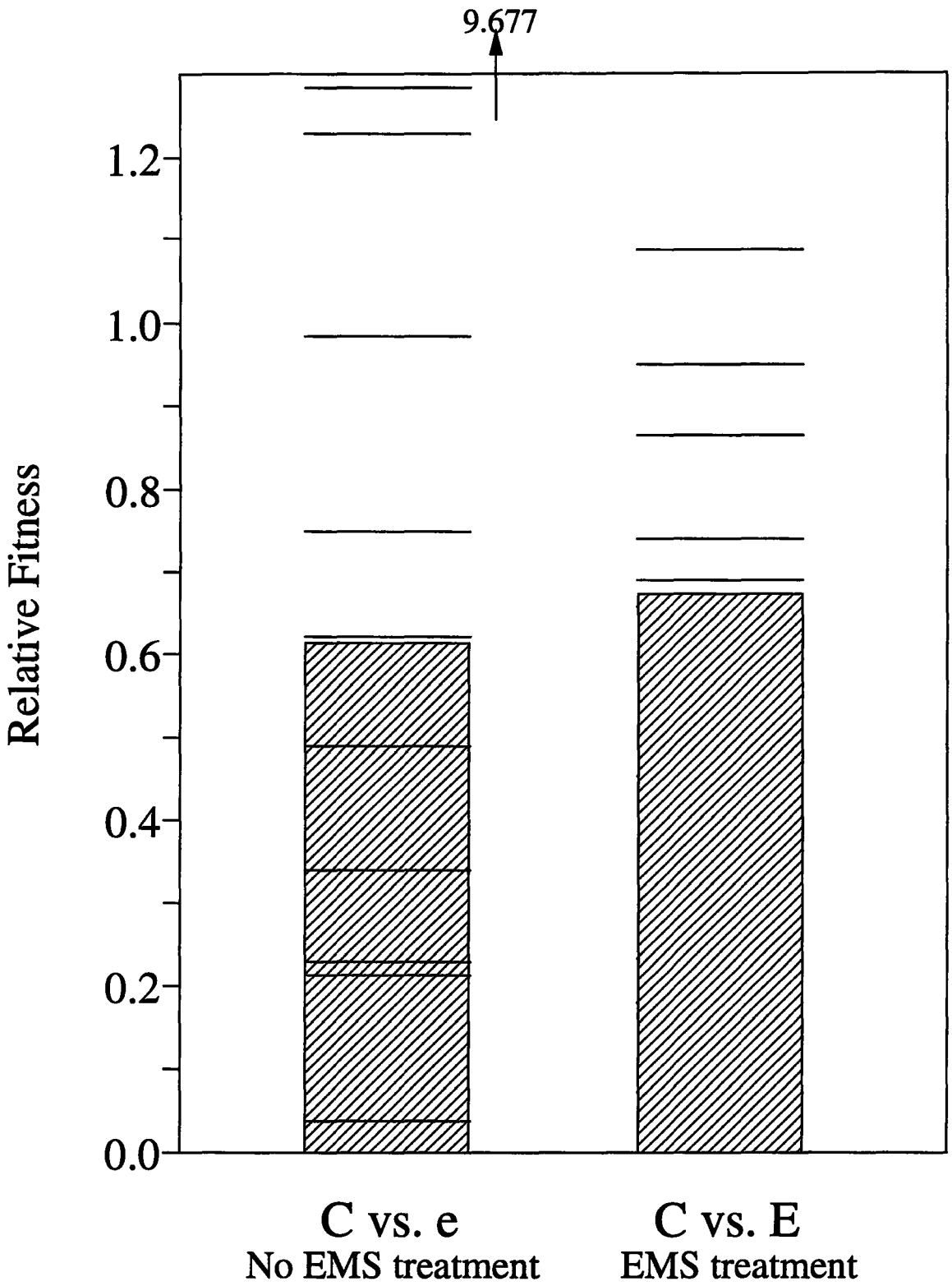
Strains	EMS	Proportion of ara^+ vs. ara^-						Time
C^+_{2b}/e^-_{3b}	-	0.211	(294)	0.050	(121)	0.034	(175)	0
		0.016	(297)	0.018	(354)	0.048	(62)	1
C^+_{2b}/E^-_{3b}	+	ND		0.249	(129)	0.257	(83)	0
		0.983	(129)	0.006	(341)	0.031	(97)	1
e^+_{3b}/C^-_{2b}	-	0.162	(136)	0.122	(84)	0.291	(136)	0
		0.644	(130)	0.829	(198)	0.734	(179)	1
E^+_{3b}/C^-_{2b}	+	0.611	(202)	0.765	(126)	0.808	(176)	0
		0.978	(256)	0.964	(181)	0.965	(258)	1

Table 4.2: Comparison of the relative fitness (W) of two strains following an overnight competition fitness assay. The relative fitness (W) was measured as the ratio of the number of doublings for the EMS adapted "E" and the control "C" cell lines (Travisano 1997). The entries with 0 were experiments where no ara^+ cells were found and hence the precise fitness value can not be calculated. Entries with "ND" indicate that the corresponding experiment was not carried out.

Strains	EMS	Fitness of ara^+ vs. ara^-			Mean Fitness	S.D
C^+_{1a}/e^-_{1a}	-	9.947	11.344	7.741	9.677	1.817
C^+_{1a}/E^-_{1a}	+	0.239	0.847	0.891	0.659	0.364
e^+_{1a}/C^-_{1a}	-	1.344	1.318	1.308	1.323	0.019
E^+_{1a}/C^-_{1a}	+	3.294	2.147	1.154	2.198	1.071
C^+_{1a}/C^-_{1a}	-	0.306	4.500	11.308	5.370	5.553
C^+_{1a}/C^-_{1a}	+	0.060	0.284	0.277	0.207	0.127
e^+_{1a}/e^-_{1a}	-	1.093	1.166	ND	1.104	0.052
E^+_{1a}/E^-_{1a}	+	0.606	0.217	0.564	0.462	0.214
C^+_{1b}/e^-_{1b}	-	1.007	1.164	1.525	1.232	0.266
C^+_{1b}/E^-_{1b}	+	0.625	0.753	ND	0.689	0.091
e^+_{1b}/C^-_{1b}	-	0.573	1.954	2.310	1.612	0.918
E^+_{1b}/C^-_{1b}	+	1.443	1.046	0.985	1.158	0.249
C^+_{1b}/C^-_{1b}	-	2.259	3.103	3.765	3.042	0.755
C^+_{1b}/C^-_{1b}	+	1.409	1.015	1.212	1.212	0.197
e^+_{1b}/e^-_{1b}	-	1.938	1.244	2.048	1.743	0.463
E^+_{1b}/E^-_{1b}	+	0.554	0.381	0.580	0.505	0.108
C^+_{2a}/e^-_{2a}	-	0	0	0	0	0
C^+_{2a}/E^-_{2a}	+	ND	ND	ND	ND	ND
e^+_{2a}/C^-_{2a}	-	1.259	0.807	0.984	1.017	0.228
E^+_{2a}/C^-_{2a}	+	0.521	1.229	11.112	4.287	5.921
C^+_{2a}/C^-_{2a}	-	0.183	0.385	0.246	0.271	0.103
C^+_{2a}/C^-_{2a}	+	1.347	1.259	1.602	1.403	0.178
e^+_{2a}/e^-_{2a}	-	0.643	1.426	0.579	0.883	0.472
E^+_{2a}/E^-_{2a}	+	1.652	1.232	1.376	1.420	0.213

Strains	EMS	Fitness of ara^+ vs. ara^-			Mean Fitness	S.D
C^+_{2b}/e^-_{2b}	-	0	0.034	0.041	0.038	0.005
C^+_{2b}/E^-_{2b}	+	0	0	ND	ND	ND
e^+_{2b}/C^-_{2b}	-	2.331	4.763	1.716	2.937	1.611
E^+_{2b}/C^-_{2b}	+	0.869	1.315	0.973	1.052	0.233
C^+_{2b}/C^-_{2b}	-	0.828	0.078	0	0.453	0.530
C^+_{2b}/C^-_{2b}	+	0.811	1.210	1.162	1.061	0.218
e^+_{2b}/e^-_{2b}	-	0.178	1.079	0.374	0.544	0.474
E^+_{2b}/E^-_{2b}	+	0.342	1.180	1.534	1.019	0.612
C^+_{2a}/e^-_{3a}	-	0.204	1.016	0.246	0.489	0.457
C^+_{2a}/E^-_{3a}	+	1.104	0.819	1.348	1.090	0.265
e^+_{3a}/C^-_{2a}	-	9.222	1.390	3.408	4.673	4.066
E^+_{3a}/C^-_{2a}	+	ND	3.336	7.767	5.551	3.133
C^+_{2b}/e^-_{3b}	-	0.076	0.360	1.412	0.616	0.704
C^+_{2b}/E^-_{3b}	+	ND	0.024	0.121	0.073	0.069
e^+_{3b}/C^-_{2b}	-	3.975	6.795	2.522	4.431	2.173
E^+_{3b}/C^-_{2b}	+	1.601	1.260	1.194	1.352	0.218

Figure 4.1: Comparison of the relative fitness (W) of two strains following an overnight competition fitness assay. The relative fitness (W) was measured as the ratio of the number of doublings for the EMS adapted "E" and the control "C" cell lines (Travisano 1997). The bar indicates the measure of the median relative fitness of the "C" control cell lines relative to the "E"/"e" adapted cell lines. A value of 1 indicates that the two clones have equal fitness; a value greater than 1 indicates that the control "C" has adapted evolutionarily: while a value less than 1 indicates that the adapted "E"/"e" cell line has adapted evolutionarily. Three replicate competition experiments were conducted for each cell line-treatment and arabinose marker state competition. Each replicate fitness estimate is treated as three independently equally accurate measurements.


9.677
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Fitness
C vs. e
No EMS treatment
C vs. E
EMS treatment

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Chapter 5

DNA Sequence Analysis

5.1 Introduction

It is suggested that a key factor in the ability of microbial populations to survive diverse environments is their large population sizes. Bacterial populations often face many environmental growth inhibiting challenges and as a consequence they have evolved survival mechanisms that enable them to cope and prevail against extreme challenges. Since these survival mechanisms are dependent upon the appropriate genetic variables it has been suggested that the organization of these genetic variables may be modulated by biochemical complexes that respond to physiological inputs and thereby serve an adaptive function (Lenski 1995). These biological complexes may be up-regulated during times of starvation or other stresses and as a result increase the mutation frequency and promote processes such as gene shuffling, duplication and

dispersal of gene sequences or amplification of repetitive DNA. The combination of an increased mutation frequency and large population size of microorganisms allow rare beneficial mutations to occur that permit survival and further propagation of the resistant individuals in the population. Studies indicate that often these genetic changes are biased towards adaptive mutations (Lenski 1995) leading to alternative metabolic pathways that are otherwise silenced or down regulated, or special pathways for enhanced repair or mutation (Koch 1993). The integrity of genetic information contained in cellular DNA is highly dependent upon the effectiveness of the DNA repair systems in an organism (Lehmann and Karran 1981). As a result organisms have a variety of repair mechanisms to combat the damage often induced by radiation or environmental agents.

In *E. coli* O^6-alkylguanine repair is mediated by two types of alkytransferases Ogt, a constitutively expressed gene product (19-kDa) and the induced *ada* gene product (37-kDa) (Roldan-Arjona *et al.* 1994). The adaptive response mechanism was first observed in a study conducted by Samson and Carins in 1977. Upon continuous exposure of *E. coli* to low doses of the alkylating agent MNNG (N-methyl-N'nitro-N-nitrosoguanidine), *E. coli* cells developed significant resistance to the mutagenic and lethal effects of higher lethal challenge doses of MNNG. This inducible repair mechanism is distinct from UV-induced error-prone SOS repair since it is error-free allowing increased survival at no cost to the cell and a significantly reduced mutation frequency compared to non-adapted cells (Lindahl *et al.* 1988; Samson and Cairns 1977). Studies indicate this repair process to be independent of *recA* and *lexA* genes and not inducible by UV or 4-nitroquinoline 1-oxide (Jeggo *et al.* 1977;

Schendel *et al.* 1978). In *E. coli* the inducible O^6-methylguanine-DNA methyltransferase I (O^6MGT I) or Ada protein acts to reduce alkylation damage by transferring the methyl group from the alkylated DNA to its own cysteine residue, leaving behind an unmodified guanine base (Demple *et al.* 1982; Lindahl *et al.* 1988). The methylated Ada molecule is quite stable, it is not actively demethylated and as a result is inactivated and expended in each reaction (Friedberg, Walker and Wolfram 1995). Ada contains two distinct methyl acceptor sites, one for methylphosphotriesters and one for O^6-methylguanine in the C-terminal (Cys-321) and the N-terminal (Cys 69) regions of the protein respectively (Lindahl *et al.* 1988). Characterization of the Ada protein has revealed a dual role, acting as a regulatory protein in addition to a DNA transferase. Self methylation of this protein converts it into a strong transcriptional activator for itself and three other genes *alkA*, *alkB*, and *aidB* involved in this inducible response (Sedgwick and Vaughan 1991; Vaughan *et al.* 1991). The Ada protein binds to a promoter region of the *ada/alkb* and *alka* genes. The binding sites of the *ada/alkb* and *alk* genes contain a common promoter region (AAANNAAAGCGCA) termed the "Ada Box" (Lindahl *et al.* 1988; Nakamura *et al.* 1988; Landini and Volkert 1995; Friedberg, Walker and Wolfram 1995). The ogt gene product, 0^6-methylguanine DNA methyl transferase II (O^6MGTII) is constitutively expressed and is responsible for repair of the majority of the alkylated lesions with a distinct preference for O^4-methylthymine, a product common to alkylating and bulky ethylating agents (Takano, Nakamura and Sekiguchi 1991; Friedberg, Walker and Wolfram 1995). It has been suggested that this repair mechanism is limited in its repair capacity and it is when this constitutive system can not repair the damage rapidly enough, that the adaptive response mechanism is induced (Schendel *et al.*

1978). The nucleotide sequence of *ogt* is distinct from the *ada* gene however, the translated amino acid sequences of these two transferases reveal extensive regions of homology. (Takano, Nakamura and Sekiguchi 1991; Friedberg, Walker and Wolfram 1995).

In previous studies long-term adapted cell lines continually exposed to low doses of the alkylating agent Ethyl methane sulfonate (EMS) for over 3000 generations cell lines have demonstrated significantly enhanced resistance to the lethal effects and a higher tolerance to the mutagenic effects of large challenge doses of EMS (Section 2) and enhanced survival upon challenge with MNNG (N'-methyl-N'-nitrosoguanidine) (Section 2 and 3). Despite this genetic adaptation to the effects of alkylating agents, attempts to induce the adaptive response mechanism in these same long-term genetically adapted cell lines were unsuccessful suggesting that either the adaptive concentrations were insufficient or the Adaptive response mechanism is defective in the cell lines themselves. This differential induction of the adaptive response in different *E. coli* strains has been observed in previous studies (Jeggo 1980). In most cases the ability or inability to induce mutagenic and lethal adaptation always occurs in parallel suggesting that this phenomenon is most likely a consequence of inhibition of transcriptional activation of the *ada* gene. *Salmonella typhimurium* studies indicate that the inability to induce the adaptive response is a result of weak transcriptional activation of its *ada* gene due to self methylation of the protein during pre-treatment with MNNG (Vaughan and Sedgwick 1991). Other known causes resulting in a lack of induction of this repair pathway include mutations in the "Ada box" sequence leading to the elimination of transcriptional activation of the *ada* gene (Nakamura

et al. 1988; Landini and Volkert 1995). Studies have shown that Ada protein activation in the absence of adaptive treatment with alkylating agents can occur through a variety of mutations in the coding region of ada^+. Mutations such as Cys 321 to Ala 321 in the active site or a truncated Ada protein with deletions of the "C" terminal domain lead to phenotypes that are constitutive for the adaptive response (Friedberg, Walker and Wolfram 1995). In the absence of adaptive response induction the Ogt protein alone protects organisms from the mutagenic damage caused by alkylating agents (Yamada *et al.* 1995). In this analysis *ada* and *ogt* sequences from the long-term evolved cell lines are examined as possible candidate regions for sequence changes that may be leading to the enhanced resistance observed in the "E" adapted cell lines and for possible changes in the *ada* gene sequence that could be the cause of the ineffective induction of the "Adaptive response" in both the control and adapted cell lines.

5.2 Methods

Bacterial Lines. The experiments were conducted using cell lines from an ancestral *E. coli* CSH100 (*ara* Δ (*gpt-lac*)5 mating type F′ *lacpro* A^+ B^+) clone obtained from Cold Spring Harbour Laboratory. This strain was separated into six individual lines that were then maintained by serial culture as pure lines for over 10 months. At this point in time these ancestral cell lines ("generation 0") were further propagated for an additional 24 months (over 4000 generations) for long-term adaptation experimental analysis.

Culture Conditions. Three replicates of two experimental treatment lines were established and have been maintained for approximately 3 years at 37^0C. All lines were propagated with M9 minimal medium and maintained daily by 1:100 serial dilution into 10 ml of fresh medium. The EMS adapted lines designated as "E" were treated daily with 25μl of EMS following serial dilution. The control lines designated as "C" were grown in the absence of EMS. All other conditions were identical for both the "E" and the "C" lines. In most of the experiments conducted a third cell line was established using an inoculate from the "E" EMS adapted line. This line designated "e" was grown up overnight (approximately 8 generations) in the absence of EMS in order to eliminate any physiological changes and distinguish between phenotypic acclimation and genetic modification.

Chemical Reagents. Ethyl methane sulfonate (EMS), agar and all necessary supplements were obtained from Sigma Chemical Co.

Bacterial Growth Medium. Liquid growth media consisted of minimal M9 solution supplemented with 20% glucose, 1M $MgSO_4$, thiamine and 0.5% thiamine per litre.

PCR conditions *ada* and *ogt* gene segments were amplified by PCR using the following conditions: PCR was performed using 1μl of genomic DNA extract, 8μl of $MgCl_2$, 5μl of 10x reaction buffer, 1μl of a 400μmol solution of each dNTP, 5U of AmpliTaq Gold DNA polymerase (PERKIN ELMER) and 2μl of both forward and reverse 10μM primer solutions (*ada* forward primer: 5'AAGCTTCCTTGTCAGCGAAA 3' 102bp 5' of start site, *ada* reverse primer:5'AAAAGCAAAACGCCGTAAAA 3' 81bp 3' of termination site, *ogt* forward primer: 3'AAAGTGTGAACAAGCTGGCA 5' 67

bp 5' of start site, and *ogt* reverse primer 5'GCGTGGTATCTTGTCGGTCT 3' 31bp 3' of stop). PCR cycling conditions were as follows: total 30 cycles, preheat 12 min at 95^0C, denaturation 30 sec at 95^0C, annealing 60 sec at 45^0C and synthesis for 60 sec at 72^0C with an additional extension phase for 300 sec at 72^0 following the final cycle. 5μl aliquots of PCR amplification products were separated by length in a 0.7% agarose gel stained with ethidium bromide. PCR products were purified using QIAquick Qiagen purification kits and sequencing was conducted in both directions at the MOBIX sequencing facility.

Sequence alignment "E" and "C" *ada* and *ogt* PCR products were aligned against know sequences for the *ada* and *ogt* genes obtained from Genbank. Clustal W multiple pair-wise sequence alignment was conducted for each gene utilizing a local alignment algorithm. Due to deterioration in the sequencing reactions after approximately 500-600 base pairs of sequences, 585 bases for the *ada* gene and 496 bases for the *ogt* gene were analyzed. Sequences differences were counted as significant only if the differences were found in both the forward and the reverse sequence data.

5.3 Results

Analysis of the sequence data from the *ada* gene(Figure 5.1) indicates that there are no sequence differences between the control "C" and the adapted "E" cell lines. There is however a CGC (Arg) to GCC (Ala) difference at position 411 and 412 in both evolved "C" and "E" cell lines compared to the GENBANK *ada* gene sequence. This is the only difference observed between the evolved cell lines and the GENBANK

sequence over the 585 bases analyzed.

Ogt sequence data (Figure 5.2) also indicates no sequence changes between the "C" control and the "E" adapted cell lines. As seen with the *ada* gene sequences, there are 9 sequence differences that appear in both the "C" control and the "E" adapted evolved cell lines when compared to the GENBANK sequence for the *ogt* gene. The following sequence differences were observed: position 76 CGT (Arg) to CGC (Arg), position 110 and 111 GCC (Ala) CGC (Arg), position 176 AAC (Asn) to AAT (Asn), position 206 GAT (Asp) to GAA (Glu), position 251 GCC (Ala) to GCT (Ala), position 254 ACA (Thr) to ACG (Thr), position 341 GAA (Glu) to GAG (Glu) and position 353 CTGC (Arg) to CGT (Arg).

5.4 Discussion

The *ada* and the *ogt* genes encode the induced and the constitutively active DNA methyl transeferases in *E. coli.* As such they appeared to be the most likely candidates for genetic changes responsible for the enhanced resistance to the lethal and mutagenic effects of large doses of alkylating agents in the long-term EMS adapted "E" cell line. The DNA sequences analyzed for the *ogt* and the *ada* genes for both the long-term evolved control *E. coli* population "C" and the long-term-evolved EMS adapted "E" population indicate no sequences differences between these two cell lines. Although changes in *ada* cannot be entirely ruled out since only 585 bases of the 1064 Base *ada* gene was analyzed and it is quite possible that genetic changes may have occurred downstream in the carboxyl terminal region. In the "E" adapted cell lines

the enhanced resistance to the lethal effects of the challenge EMS is quite pronounced compared to the resistance seen to the mutagenic effects of the agents therefore two other candidates that may be the tag and the *alkA* genes. Studies suggest that the mechanisms leading to killing resistance is different from that of mutagenic resistance (Jeggo 1980). This appears to be due to the type of alkylation damage incurred after exposure to large doses of alkylating agents. O^6-methylguanine is the primary mutagenic lesion caused by alkylation damage while 3-methyladenine and 3-methylguanine occur in significantly smaller amount however these are the primary lethal lesions caused by alkylation damage (Friedberg, Walker and Wolfram 1995). Following alkylation damage, most of the 3-methyadenine adducts are removed by the constitutive DNA glycosylase I also known as the *tag* gene. However, this glycosylase is insufficient to remove all alkylation damage following a large dose alkylation exposure, and 3-methyladenine and 3-methylguanine remain in the minor groove of the helix which is lethal to the cell unless excised. This repair mechanism is conducted by the inducible DNA glycosylase II. The *E. coli alka* gene is the structural gene for this enzyme also referred to as 3-methyl-adenine-DNA glycosylase (Lindahl *et al.* 1988; Landini and Volkert 1995; Friedberg, Walker and Wolfram 1995). This enzyme recognizes and excises a variety of methylated DNA bases (Lindahl *et al.* 1988; Landini and Volkert 1995). Like O^6-methylguanine-DNA methyltransferase I, *alkA* is controlled by the ada^+ locus and is considered a component of the adaptive response. There are a variety of possible causes that may have lead to the inability to induce the "Adaptive response" repair mechanism in our long-term evolved cell lines as seen in previous studies (as described in Chapter 2). Mutations in the promoter region (AAANNAAAGCGCA) termed the "Ada Box" eliminate transcriptional activation

of the *ada* gene (Nakamura *et al.* 1988; Landini and Volkert 1995; Nakamura *et al.* 1988; Friedberg, Walker and Wolfram 1995). Although the 5' promoter region of the transciptional start site could be analyzed due to sequencing deterioration, accurate sequences could not be obtained in both directions for this region 79 bases 5' to start site. However, reverse sequence in this region for the control cell line indicates a A-G transition in the "Ada Box" sequence. A point mutation in this region would result in the inability to initiate transcription of Ada and would explain the inability to induce the Adaptive Response in the control cell lines. Further upstream sequences would have to be obtained to confirm mutations in this binding region. There are no observed sequences differences for 585 bases of the *ada* gene including the Cys 69 active site (CCC) which could account for the inability to induce the adaptive response in the evolved cell lines. This Cys 69 active site is one of the two acceptor cysteines in the amino terminal of the Ada protein involved in the removal of simple alkyl groups from DNA (Friedberg, Walker and Wolfram 1995). The alkylation of this amino acid converts Ada into a strong transcriptional activator of itself and the *aidB*, *alkB* and *alkA* Adaptive Response genes (Friedberg, Walker and Wolfram 1995). The carboxyl terminal 67 amino acids of Ada is also required for the activation and negative modulation of Ada (Friedberg, Walker and Wolfram 1995). Studies have shown that Ada protein activation in the absence of adaptive treatment with alkylating agents can occur through a variety of mutations in the coding region of *ada*. Mutations such as a Cys 321 to Ala 321 in the active site or a truncated Ada protein with a 12% deletion of the "C" terminal domain lead to phenotypes that are constitutive for the adaptive response (Friedberg, Walker and Wolfram 1995). Due to sequencing size limitations sequences encoding the carboxyl domain of the Ada protein were not analyzed.

Significant DNA sequence changes in some cases resulting in amino acid changes were observed in both the "C" control and the "E" adapted cell lines relative to the *E. coli*K-12 GENBANK sequence in both the *ogt* and the *ada* DNA sequences. In the *ada* gene only one change difference is noted leading to a Arg to Ala amino acid change. In the *ogt* gene sequence a total of nine differences were resulting in 2 amino acid changes were observed. There are three possibilities to explain these observations; the first is an inaccurate sequence in the GENBANK database for the *ada* gene. Second, is that parallel genetic changes occurred in both the "C" control and the "E" adapted cell lines over the 4000 generations of long-term culturing. Third, and the most probable is that these sequence differences were present in the CSH100 *E. coli* strain used to generate these populations. This possibility could be easily tested by sequence analysis of the *ogt* and the *ada* genes from frozen CSH100 founding strain samples.

Figure 5.1: ClustalX multiple sequence alignment for the upstream nucleotide sequence of the *ada* gene. Sequences were obtained from Genbank and from *E.coli* strains "C1" (generation 3400) and E1 (generation 3400)

CLUSTAL X (1.64b) MULTIPLE SEQUENCE ALIGNMENT

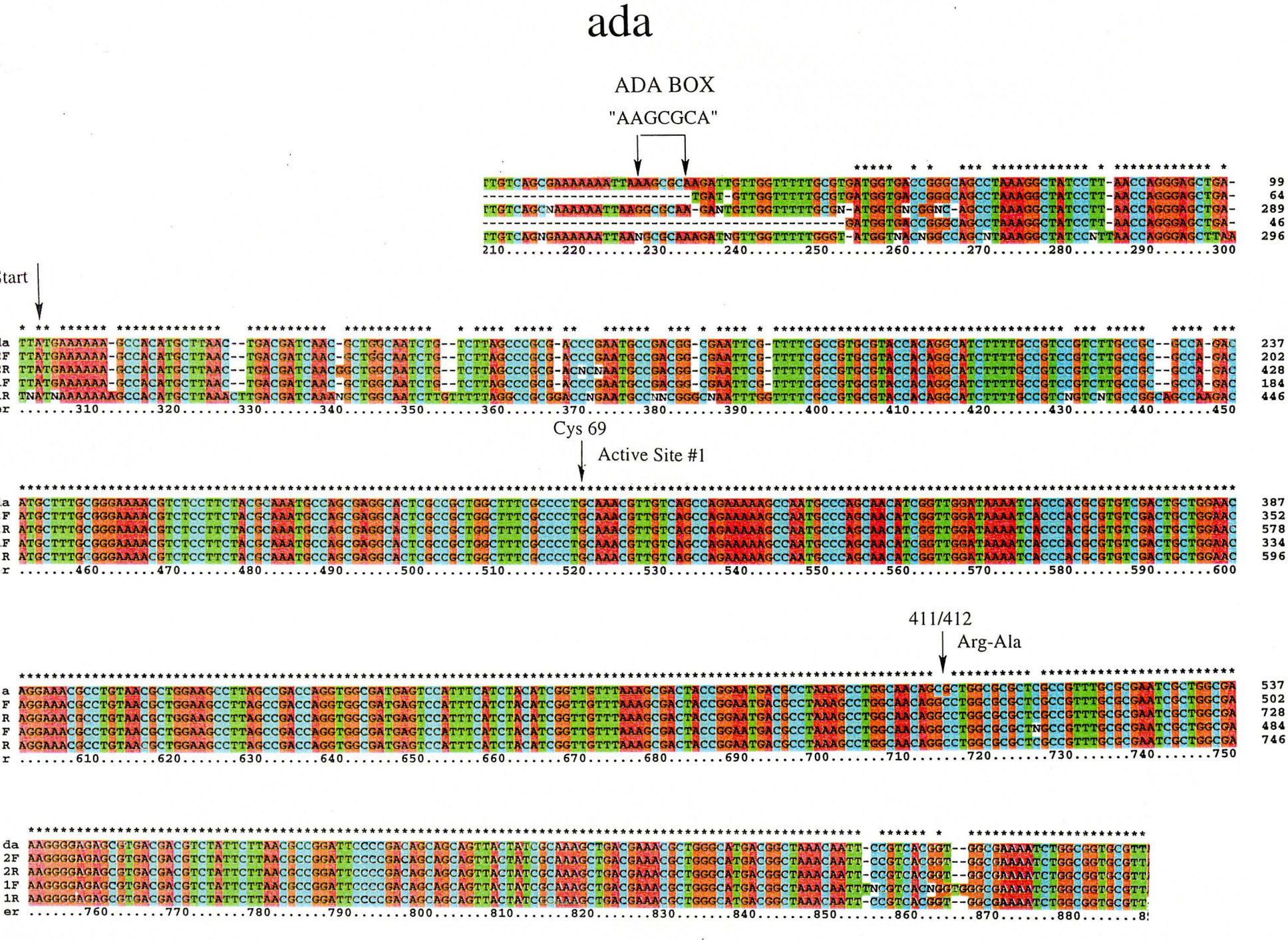
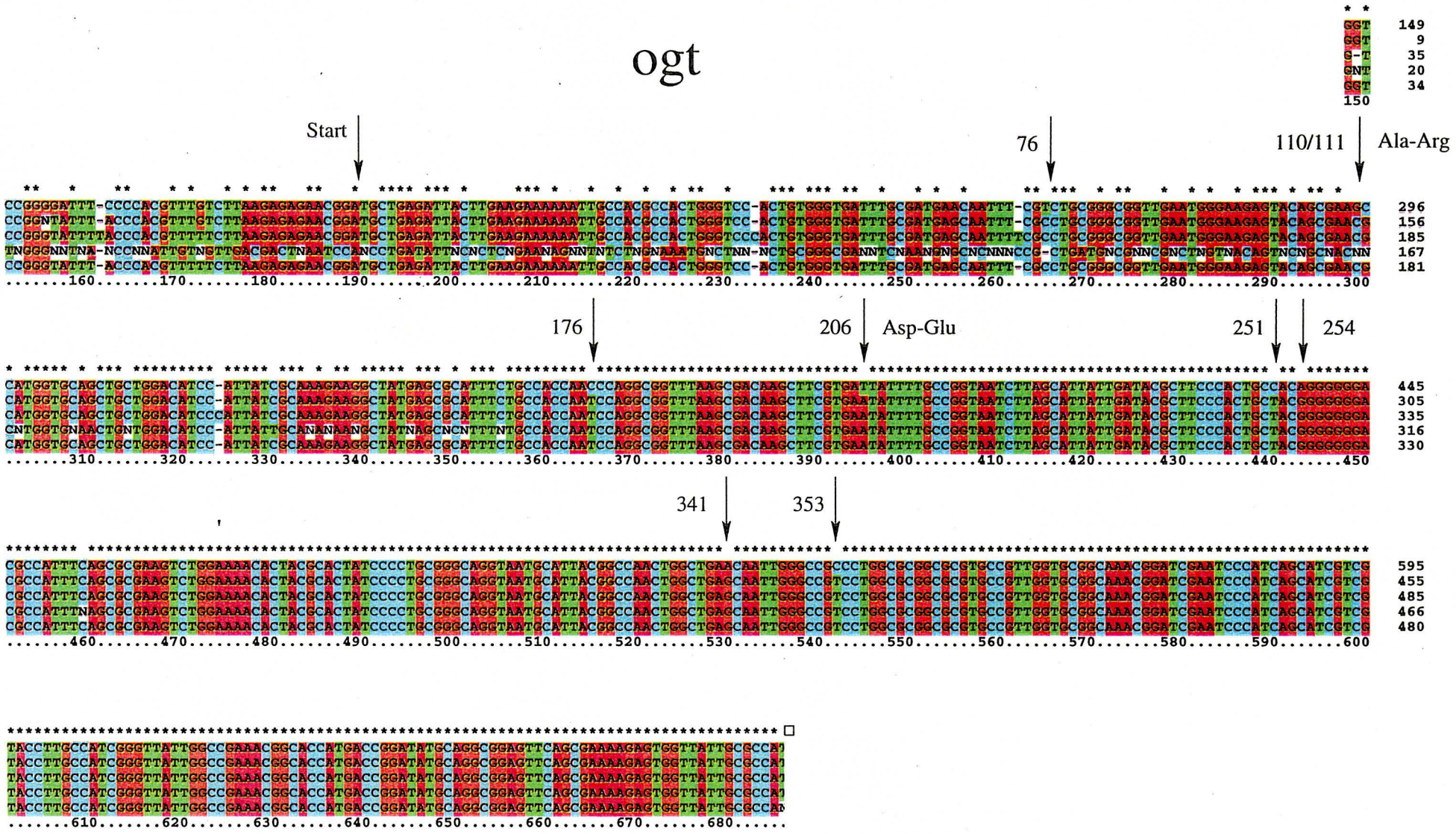


Figure 5.2: ClustalX multiple sequence alignment for the upstream nucleotide sequences of the *ogt* gene. Sequences were obtained from Genbank and sequenced from *E. coli* strains C2(generation 3400) and E1(generation 3400)

CLUSTAL X (1.64b) MULTIPLE SEQUENCE ALIGNMENT



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Chapter 6

Summary

Replicate isogenic populations of *E. coli* were propagated and maintained for over 4000 generations in order to investigate the adaptation of *E. coli* to increased levels of the mutagen Methanesulfonic acid ethyl ester (EMS).

General characterization of the long-term evolved cell line reveals that many genetic changes had taken place over the course of 4000 generations of bacterial propagation. These changes affected antibiotic resistance, β-galactosidase levels, REP element fingerprints and lethal and mutagenic tolerance to large doses of alkylating agents. Mutation frequency and survival assays conducted in this investigation strongly suggest that prior long-term low dose exposure to EMS results in significantly higher levels of resistance to the lethal and the mutagenic effects of larger challenge doses of EMS compared to long-term evolved control "C" cell lines. In addition, both survival and inhibition disk assays suggest a cross adaptive response between EMS

and MNNG, showing enhanced survival and reduced growth inhibition zones in cells adapted to EMS and challenged with MNNG.

Fitness estimates from preliminary competition experiments suggest an overall higher relative fitness for the "E"/"e" EMS adapted cell lines compared to the "C" control cell lines in both the presence of EMS. Unexpectedly the fitness estimates also suggest a higher relative fitness for the "E"/"e" EMS adapted cell lines in the absence of EMS treatment, suggesting that the EMS specific adaptation may also result in improved fitness in novel environments. These relative fitness estimates support the survival and mutation frequency analysis suggesting that the "E"/"e" adapted cell lines has significantly higher levels of resistance to the mutagenic and lethal effects of EMS. The high variability among replicate fitness estimatesmay be a consequence of inacurate discrimination between ara^+ and ara^- colonies possibly due to diminished colour reactions., therefore the results from the competition experiments are inconclusive.

Attempts to induce the adaptive response repair pathway were not successful in either the control "C" or the EMS adapted "E"/"e" cell lines suggesting that the genetic adaptation resulting in increased resistance to the lethal effects of EMS and MNNG could likely be a result of enhanced activity of the constitutive transferase Ogt and the constitutive glycosylase Tag; the two enzymes responsible for the majority of the repair of O^6-methylguanine and 3-methyladenine/3-methylguanine respectively in organisms unable to induce the adaptive response (Yamada *et al.* 1995). Induction of the adaptive response has been shown to be strain sensitive (Jeggo 1980) and the inability to induce the response is usually a consequence of transcriptional inhibition

caused by self methylation (Vaughan *et al.* 1991; Sledziewska-Gojska 1995) or mutations in the "*ada* Box" promoter region (Lindahl *et al.* 1988; Nakamura *et al.* 1988; Landini and Volkert 1995; Friedberg, Walker and Wolfram 1995).

The *ada* and the *ogt* genes encode the induced and the constitutively active DNA methyl transeferases in *E. coli.* As such, they initially appeared to be the most likely candidates for genetic changes responsible for the enhanced resistance to the lethal and mutagenic effects of large doses of alkylating agents in the long-term EMS adapted "E"/"e" cell line. The DNA sequences analyzed for the *ogt* and the *ada* genes for both the long-term evolved control *E. coli* population "C" and the long-term-evolved EMS adapted "E"/"e" population indicate no sequences differences between these two cell lines. Although changes in *ada* cannot be entirely ruled out since only 585 bases of the 1064 base *ada* gene was analyzed and it is quite possible that genetic changes may have occurred downstream. In the "E"/"e" adapted cell lines the enhanced resistance to the lethal effects of the challenge EMS is quite pronounced compared to the observed mutagenic resistance. The constitutive DNA glycolsylase *tag* gene and the induced DNA glycosylase *alkA* genes are the two genes primarily involved in adaptation to prevent killing and would therefore be strong additional candidates for adaptive genetic changes. Significant DNA sequence changes in some cases resulting in amino acid changes were observed in both the "C" control and the "E"/"e" adapted cell lines relative to the *E. coli* GENBANK sequence in both the *ogt* and the *ada* DNA sequences. This is most likely due to sequence differences that were present in the CSH100 *E. coli* strain used to generate these populations.

Previous studies have primarily observed *E. coli's* ability to phenotypically accli-

mate over very short time intervals to EMS. This analysis has shown that long-term genetic adaptation to low doses of EMS results in enhanced resistance to both the lethal and the mutagenic effects of larger challenge doses of EMS.

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