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The Characterization of an *Escherichia coli* Strain Deficient in Very Short Patch Mismatch Repair

Sonia Ruiz

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ABSTRACT

The Characterization of an *Escherichia coli* Strain Deficient in Very Short Patch Mismatch Repair

Sonia Ruiz

In many strains of *Escherichia coli*, the product of the *dcm* gene methylates the internal cytosine in the DNA sequence 5'-CC(A/T)GG-3'. Deamination of 5-methylcytosines results in T·G mismatches. If unrepaired, these mismatches can lead to C to T transition mutations. The very short patch (VSP) repair process in *E. coli* counteracts the mutagenic process by repairing the mismatches in favor of the G-containing strand.

A genetic selection system capable of isolating *E. coli* VSP mutators was designed and one such mutator, SPR112, was isolated. Like *vsr*, the only gene which has been unequivocally linked to VSP repair to date, the mutation maps at 43 minutes. However, this mutation is not complemented by *vsr* and various experimental results suggest that the mutation in SPR112 is not in *vsr* but in another gene also required for VSP repair. This mutator locus has been designated *mutG*.

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First and foremost, I would like to thank my supervisor, *Dr. Claire Cupples*, for her excellent advice and continuous support throughout the entire duration of the project. Thank you *Dr. C.* for always being there.

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Special thanks to my good friend *Skidoo* for listening to the daily "what didn't work " update. *P.S.* I didn't mean to bore you; it wasn't my fault the *same* experiment failed *so many times*!

It would be too long to individually list all the people within the department (faculty, staff, and students) whose friendship made Concordia University a good place to be. You know who you are. Thanks guys.

Last but not least, I wish to extend my sincere gratitude to my mother. *Mom* your encouragement and wisdom made a difference.

Although I did not know any of these people personally, I wish to dedicate this thesis to the victims of the fatal shooting which occurred here at Concordia University on August 24th, 1992, during the final days of its completion: *Matthew Douglass, Michael Hogben,* and *Jann Saber.*

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INTRODUCTION

Base pair mismatches arise continually in the DNA of an organism. Repair mechanisms for the correction of base pair mismatches in DNA are known to exist in bacteria, fungi, and in higher eukaryotes, including humans (GRILLEY *et al.*, 1990). In this thesis, the term mismatch correction is used to refer to processes in which recognition of a mismatched base pair elicits a specific response resulting in its repair. It is in the prokaryote *Escherichia coli* (*E. coli*) that most of the mismatch correction enzymology has been developed. *E. coli* is the organism of choice because it is easy to manipulate genetically, has a number of simple assay systems, and has numerous functional homologies with eukaryotes. Consequently, all the research described in this thesis was done in *E. coli* and discussions focus on its mismatch repair systems; whenever possible, comparisons to repair mechanisms in other organisms are included. *E. coli* has a number of mismatch repair mechanisms; the purpose of this research project was to elucidate the mechanism of one such DNA correction process, the very short patch (VSP) mismatch repair system. Before proceeding to a detailed discussion on the work that was done, mismatches, how they can arise, and the various repair mechanisms that have been characterized to date are discussed.

Complementary Base Pairing and Replication Fidelity in DNA

DNA consists of two very long antiparallel helical chains of deoxyribonucleotides wound around a common central

axis. The two chains of the double helix are joined by hydrogen bonds (H-bonds) between specific pyrimidine-purine base pairs. Chemically, the most stable configuration occurs when a thymine pairs with an adenine (T·A), and cytosine pairs with a guanine (C·G), so that one strand of the double helix is the complement of the other (Villee, et al., 1985).

The fidelity of DNA replication appears to be controlled by two enzymatic mechanisms: (1) base selection by the DNA polymerase and associated proteins; and (2) the proofreading function of the DNA polymerase. Postreplicative mismatch repair systems correct lesions that escape proofreading or that arise spontaneously after replication. In *E. coli*, the DNA replication fidelity is about 10^{-9} - 10^{-10} errors per replicated nucleotide (DRAKE, 1969 cited in SCHAAPER and RADMAN, 1989). This is compatible with the estimated base-insertion fidelity of the α-subunit of the polymerase (10^{-5}) (for review, see Loeb and Kunkel, 1982) combined with the calculated value for proofreading of the ϵ-subunit of the polymerase (10^{-2} - 10^{-3}) and post-replicative mismatch repair (10^{-2} - 10^{-3}, implied from the *mutHLS* mutator effect only) (RADMAN and WAGNER, 1986; SCHAAPER and DUNN, 1987a; SCHAAPER and RADMAN, 1989).

Mismatches

A mismatch can be defined as any noncomplementary base pair in double-stranded DNA. For the purpose of this thesis

the term mismatch will be used to refer to transition (T·G and C·A) and transversion (C·C, T·T, C·T, A·A, G·G, and A·G) mismatches. The research focuses on T·G mispairs.

How Can a Mismatch Arise?

Mismatched base pairs can occur spontaneously *in vivo* as a result of: (1) errors in DNA replication or repair synthesis, when the polymerase fails to insert a base that complements the template strand; (2) recombination between homologous but not identical DNA sequences; and (3) the chemical modification of bases such as deamination of 5-methylcytosine to form T·G mispairs. With respect to this research, it is the third process which is of interest. Compared to the first two processes described, this process is static and specific; it does not occur during DNA replication or recombination and it gives rise to T·G mismatches only. In *E. coli*, the product of the deoxycytosine methylase gene, *dcm*, methylates the fifth carbon of the second C in the sequence CCAGG. These 5-methlycytosines ($^{5\text{-me}}$C) are unstable; some spontaneously deaminate becoming thymines resulting in T·G mismatches.

The repair of such mismatched base pairs is essential to maintain genetic stability, and ultimately, to maintain species survival. Consequently, repair mechanisms or systems which recognize and correct these base pairing errors, have evolved. For a recent review of DNA repair systems, refer to GRILLEY *et al.*, 1990.

HOLLIDAY (1964) was the first to propose the concept of DNA mismatch repair mechanisms. *E. coli* has a number of DNA mismatch correction processes. Characterized *E. coli* repair systems can be classified into three categories: (1) ***Pre-replication or preventive***, (2) ***Polymerase related***, and (3) ***Post-replication***. The following is a comparative review of the mechanisms of the best characterized correction systems in *E. coli*. However, new systems continue to be identified. For example, two mutator loci, *mutA* and *mutC* that stimulate A·T to T·A transversions have been identified in *E. coli* (MICHAELS *et al.*, 1990); investigators suggest that *mutA* and *mutC* may be involved in the same correction system, but these have not been characterized further.

The isolation and characterization of the diverse *E. coli* mutator strains - cells which have a high spontaneous mutation rate due to a defect in a repair gene - has been highly revealing as to the molecular strategies that ensure the replication fidelity of bacterial DNA.

Class 1: Prereplication or Preventive

The term prereplicative or preventive is used to refer to various processes which serve to minimize polymerase error during DNA replication.

The MutT Repair Process

Cells deficient in *mutT* have elevated levels of A·T to

C·G transversions (YANOFSKY, 1966). *MutT* was cloned by AKIYAMA and coworkers and BHATNAGAR and BESSMAN (AKIYAMA *et al.*, 1989; BHATNAGAR and BESSMAN, 1988). *MutT* plays a preventive role in avoiding dG·dA mispairings in replicating DNA. In a cell's nucleotide pool, some dGTP (deoxyguanylate 5'-triphosphate) spontaneously oxidizes, becoming 8-oxo-7,8-dihydro-2'-dGTP (8-oxodGTP) (MAKI and SEKIGUCHI, 1992). The DNA polymerase III does not seem to discriminate against this oxidized form of the guanine nucleotide; during DNA replication, the polymerase inserts 8-oxodGTP opposite dA residues as often as dC residues (LOVELESS, 1969, cited in MAKI and SEKIGUCHI, 1992; LOECHLER *et al.*, 1984). Further, the editing function of the polymerase does not replace this misincorporated base (AKIYAMA *et al.*, 1989). *MutT* codes for a 15-kDa dGTPase, which only hydrolyzes 8-oxodGTP to dGMP (deoxyguanylate 5'-monophosphate). It is by removing the oxidized form of the guanine nucleotide from the cell's nucleotide pool that the protein product of *mutT* protects DNA from oxidation damage (MAKI and SEKIGUCHI, 1992). In other words, the *mutT* protein prevents dG·dA mispairing in replicating DNA by hydrolyzing 8-oxodGTP, a potent mutagenic substrate.

Class 2: Polymerase-Related

In *E. coli*, three DNA polymerases have been characterized: DNA polymerase I, DNA polymerase II, and DNA polymer-

ase III. Polymerase III is necessary for DNA replication (COX, 1976). All three polymerases can extend growing daughter strands by adding nucleotides from the 5' to the 3' end. The discriminating function of the α-subunit of the polymerase, product of the *dnaE* gene, itself serves to promote complementary base pairing.

Also associated with many DNA polymerases is an editing function. The ϵ-subunit of the *E. coli* DNA polymerase III, encoded by *dnaQ* or *mutD*, has a 3' to 5' exonuclease function that can identify and remove incorrectly paired bases immediately following biosynthesis.

Polymerases with properties similar to *E. coli* polymerase I and III have been characterized in *Bacillus subtilis* and in *Micrococcus luteus*. DNA fidelity in eukaryotes is comparable to that of *E. coli*. However, characterized DNA polymerases do not possess the 3' to 5' exonuclease activity, and do not recognize mismatched 3' termini; it has been proposed that *in vivo*, the proofreading function exists as separate subunits which are associated with the replicating machinery (COX, 1976).

Class 3: Post-Replication

In non-replicating DNA, guanines that oxidize spontaneously *in situ* or that escaped *mutT* are removed by the *mutM* repair process.

The MutM Repair Process

Some dG, paired with dC, can spontaneously oxidize to 8-oxo-7,8-dihydro-2'-dG (8-oxodG). The *E. coli mutM* repair process removes this oxidized guanine restoring the complementary G·C base pair. However, *mutM*$^-$ strains do not remove this 8-oxodG. In the next round of replication, the polymerase inserts either dA or dC residues opposite 8-oxodG. A·8-oxodG mismatches should be repaired by the *mutY* repair system which will be discussed later. However, in a *mutM*$^-$, *mutY*$^-$ strain, the A·8-oxodG mismatch is not repaired; consequently, the next round of DNA replication generates G·C to T·A transversions (CABRERA *et al.*, 1988). The nucleotide sequence of the cloned *mutM* is identical to the previously isolated *fpg* (formamide pyrimidine glycosylase) gene (BOITEUX *et al.*, 1990; TCHOU *et al.*, 1991). *Fpg* codes for a formamidopyrimidine (Fapy) DNA glycosylase; it catalyzes the release of imidazole ring-opened forms of guanine and adenine from alkylated or irradiated polynucleotides and DNA (TCHOU *et al.*, 1991). Its function in repair is to excise oxidized guanine inserted opposite dC so as to prevent mismatches in subsequent replications.

Therefore, *E. coli* seems to have at least two mechanisms which are involved in the protection of DNA from oxidation damage: MutT protein functions at the base selection level, and the MutM (Fpg) protein functions at the DNA level (MAKI and SEKIGUCHI, 1992).

Mismatches arising from biosynthetic errors that escape

proofreading are subject to post-replication mismatch correction. To date, three *E. coli* mispairing repair mechanisms that have been characterized fall in this category: the *dam*-directed mismatch repair system, *mutY*, and very short patch (VSP) repair.

(i) The Methyl-Directed Mismatch Repair System

The *dam*-directed mismatch repair system can correct a wide range of improper base pairings which occurred during DNA replication or recombination (KRAMER *et al.*, 1984; RAPOSA and FOX, 1987). Essentially, the system functions to minimize mutations and to maximize recombination fidelity (GRILLEY *et al.*,1990). However, transition mismatches tend to be better repaired than transversion mismatches (KRAMER *et al.*, 1984). The products of genes *mutH*, *mutL*, *mutS*, and *mutU* (or *uvrD*), the single stranded DNA binding (SSB) protein, and the DNA polymerase III holoenzyme (LU *et al.*, 1983; PUKKILA *et al.*, 1983) are required for the repair process to function. Results of *in vivo* and *in vitro* experiments elucidated the mechanism of the *dam*-directed mismatch repair system (see ***FIGURE 1***).

During DNA replication, the DNA polymerase unwinds both strands and these are used as templates. Adenines at d(GATC) sequences of the template strands, are methylated. However, in newly synthesized strands, adenines are not yet methylated. The state of adenine methylation at d(GATC) sequences directs the repair to the newly synthesized strand (LU *et*

al., 1983). The repair process occurs preferentially on unmethylated strands of hemimethylated heteroduplexes (LANGLE-RCUAULT *ET AL.*, 1987; WELSH *et al.*, 1987). The product of the *mutS* gene, a 97-kDa protein binds to the mismatch. The 70-kDa protein product of *mutL* binds to the *mutS*-heteroduplex complex. Assembly of this repair complex activates the *mutH* gene product (K. AU,K. WELSH, and P. MODRICH in preparation, cited in GRILLEY *et al.*, 1990). The product of *mutH* is involved in directing the repair to the newly synthesized strand; it incises immediately 5' of the dG in the d(GATC) sequence in the unmethylated or hemimethylated strand. The DNA helicase II, product of *mutU*, unwinds the DNA from the single stranded break to just beyond the mismatch. The *exonuclease* I hydrolysis of the single-stranded DNA to mononucleotides is enhanced by the adhesion of the 18-kDa SSB protein to the single stranded DNA. The resulting gap is filled by the DNA polymerase III holoenzyme and the nick is sealed by DNA ligase.

In *E. coli*, methylation of the newly synthesized strand lags behind the replication fork. The delay with which methylation occurs following replication determines the time the methyl-directed repair system has to recognize and repair replication errors and consequently, affects the efficiency of mismatch repair. *Dam* methylase overproducer strains are mutators (HERMAN and MODRICH, 1981). Compared to wild type strains methylation may occur more rapidly in these strains thus shortening the time available for mis-

match repair (RADMAN and WAGNER, 1986).

SU and coworkers (1988) determined the specificity of the *dam*-directed mismatch repair mechanism. Results indicate that C·C mismatches are an extremely poor substrate for repair. Further, the methyl-directed repair system processes A·G mismatches to A·T only; these findings indicate that the correction of A·G mispairs to C·G occur largely by a pathway independent of the methylation state of the heteroduplex and does not require *mutH*, *mutL*, or *mutS* gene product. Evidence provided by RADICELLA and coworkers (1988) suggests that the removal of A in A·G (and A·C) mismatches is mediated by the product of the *mutY* locus.

In contrast to the *dam*-directed repair, the *mutY* and the very short patch repair system do not rely on the state of methylation at d(GATC) sequences, have no strand specificity, and repair only a specific mismatch.

The methyl-directed mismatch repair system has also been characterized in *Salmonella typhimurium*.

A general repair pathway similar to the *dam*-directed repair system in *E. coli* has been identified in *Streptococcus pneumoniae*; this correction process requires the *hexA* and *hexB* genes, however, single-strand breaks seem to direct the repair to the proper strand rather than hemi-methylation (CLAVERYS *et al.*, 1984).

In eukaryotes, mismatch repair is not as well understood. In *Saccharomyces cerevisiae* efficient mismatch repair requires the *PMS1*, *PMS2*, and the *PMS3* gene products. Howev-

er, methylation is unlikely to play a role in the PMS system in yeast since adenine and cytosine are not methylated in yeast DNA (HATMAN *et al.*, 1978 and PROFFITT *et al.*, 1984 cited in MANKOVICH et *al.*, 1989).

The HexB protein of *Streptococcus pneumoniae* and the PMS1 protein in *Saccharomyces cerevisiae* are homologous to the MutL protein of *Escherichia coli* and *Salmonella typhimurium* (PRUDHOMME *et al.*, 1989; MANKOVICH *et al.*, 1989).

A general repair system similar to the mismatch repair system in *E. coli* has been observed in nuclear extracts of human HeLa cells and in *Drosophila melanogaster* cell line (HOLMES *et al.*, 1990; THOMAS *et al.*, 1991). One obvious difference between *E. coli* and the two eukaryotic systems is the signal employed in strand discrimination. As in *Streptococcus pneumoniae*, a nick in the DNA of human HeLa cells and *Drosophila melanogaster* is required for efficient repair. Repair is predominant in the nicked strand (HOLMES *et al.*, 1990; THOMAS *et al.*, 1991).

(ii) The MutY Repair Process

The *mutY* repair system processes A·G and A·C mispairs to C·G and G·C, respectively. Like *mutM*, *mutY* is implicated in the reduction of G·C to T·A transversions (AU *et al.*, 1988; NGHIEM *et al.*, 1988). This repair system does not require *mutH*, *mutL*, *mutS*, or *mutU* gene products. *mutY* codes for a 36-kDa G·A specific adenine glycosylase (AU *et al.*, 1989). AU and coworkers (1989) reported that under defined

conditions, *mutY*, AP endonuclease II, DNA polymerase I, and DNA ligase are sufficient to mediate specific G·A to G·C correction with no apparent mechanism for distinguishing parental from newly synthesized strands. The repair process is as follows (see ***FIGURE 2***): the complementarity of DNA is disrupted by a G·A mismatch. The gene product of *mutY*, a G·A specific glycosylase, recognizes the mismatch and hydrolyzes the glycosylic bond which attaches the mispaired adenine to the deoxyribose. An AP endonuclease cleaves the phosphodiester backbone adjacent to the missing base. From the 3'-OH group, the DNA polymerase I resynthesizes the complementary fragment. The remaining nick is sealed by DNA ligase.

YEH and coworkers (1991) identified two enzyme systems in nuclear extracts of human HeLa cell lines that can nick at specific sites of DNA molecules containing mismatched bases; one of these systems is specific for A·G mismatches. Although there is no evidence showing that the HeLa A·G-specific excision activity is mediated by a DNA-glycosylase-AP endonuclease, the human A·G-specific enzyme is similar to the *E. coli* mutY A·G specific glycosylase. Both systems recognize and nick the A in A·G mismatches and have no requirement for Mg^{2+} or ATP (YEH *et al.*, 1991). These results suggest that an A·G specific correction process similar to the *mutY* repair system in *E. coli* exists in humans.

(iii) The Very Short Patch Repair System

When the methyl-directed mismatch repair system was

disrupted by mutations in the *mutH* or *mutU* (*uvrD*) gene, a localized repair process that is independent of the degree of methylation of adenines at d(GATC) sites became prominent (RAPOSA and FOX, 1987). Results of *in vivo* experiments suggest that this repair process requires the *mutL* and *mutS* gene products (JONES *et al.*, 1987b; LIEB, 1987; ZELL and FRITZ, 1987). Later studies revealed that this prominent repair system was very similar to the very short patch (VSP) repair system described by LIEB (RADICELLA *et al.*, 1988); it efficiently corrects G·T mismatches at the second C of the nucleotide sequence CC(A/T)GG sites in quiescent (ie. non-replicating and nonrecombining) DNA. The G·T mismatches arise from C·G base pairs and correction restores the C·G pair. As its name implies, very short patch repair involves the excision and resynthesis of a small tract of DNA (including the mismatched T) from two to ten bases long (LIEB, 1983; confirmed by JONES *et al*, 1987b). The incorrect nucleotide is thus removed and the correct one is inserted. ***FIGURE 3*** summarizes VSP repair. Very short patch repair works only at 5'CC(A/T)GG, 5'CCAG, and 5'CAGG nucleotide sequences (JONES *et al.*, 1987a; LIEB, 1985; LIEB, 1987).

How do the G·T mismatches occur? In *E. coli*, the product of the deoxycytosine methylase gene, *dcm*, methylates the second C in the sequence CC(A/T)GG to a $^{5\text{-me}}$C (MARINUS, 1984). However, some of these $^{5\text{-me}}$C spontaneously deaminate to thymines, forming a T·G mismatch at the second C of the sequence CCAGG. If VSP repair is functional, the T·G mis-

match will be repaired and the original C will be restored. However, if VSP repair is not functional, the mismatch will not be corrected and in the next round of replication, C·G to T·A transitions will occur. In other words, VSP repair is a very specific repair mechanism. It corrects only T·G mismatches to C·G (as opposed to T·A) that results from the spontaneous deamination of 5-methylcytosines in the second C in the very specific nucleotide sequence CC(A/T)GG (JONES *et al.*, 1987a). However, G·T mismatches that arise due to an error in replication should be corrected by the procfreading function of the polymerase or by the methyl-directed repair system (JONES *et al.*, 1987b).

The G·T mismatches that arise due to an error in replication should be corrected by the proofreading function of the polymerase or by the methyl-directed repair system (JONES *et al.*, 1987b).

The specific action of VSP repair on cytosine methylation sequences suggests that VSP repair has evolved to minimize the mutagenic consequences of cytosine methylation, thus conserving the genetic integrity of the cell (JONES *et al.*, 1987a; LIEB, 1991; RADMAN and WAGNER, 1986). However, as will be shown, VSP mutators (ie. cells deficient in VSP repair) are viable. So, C to T mutations at CCAGG sequences in the cell are not necessarily lethal. SOHAIL and coworkers, 1990, isolated a gene, *vsr*, which is involved in VSP repair; further, the 5' end of *vsr* overlaps the 3' end of *dcm* (SOHAIL *et al.*, 1990).

Very short patch repair has also been characterized in *Streptococcus pneumoniae* (BROOKS *et al.*, 1989).

VSP Repair in Mammalian Cells

In the DNA of higher eukaryotes, methylation of cytosines within CpG dinucleotides plays an important role in the regulation of gene expression (JIRICNY et *al.*, 1988). To maintain the differentiated state of the cell, the DNA must be protected from the effects of the spontaneous deamination of $^{5\text{-me}}C$ (RIGGS and JONES, 1983, cited in JIRICNY *et al.*, 1988).

The presence of considerable amounts of 5-methylcytosines in mammalian DNA and the high rate of spontaneous deamination of 5-methylcytosine to thymine suggests the need for a repair system analogous to the short patch repair system in *E. coli* (RADMAN and WAGNER, 1986).

A system analogous to VSP repair whereby G·T mispairs, resulting from the spontaneous hydrolytic deamination of 5-methycytosine to thymine, are preferentially repaired to G·C, has been observed in simian cells (BROWN and JIRICNY, 1987) and in human cell extracts (WIEBAUER and JIRICNY, 1989). JIRICNY and coworkers have characterized this repair process in humans and found the mechanics to be similar to that of the *mutY* correction system in *E. coli*.

A thymine glycosylase removes the deaminated base (thymine), generating an apyrimidinic (AP) site opposite the guanine (WIEBAUER and JIRICNY, 1989; WIEBAUER and JIRICNY,

1990). The single nucleotide gap, generated by the cleaving of the sugar-phosphate backbone by an AP endonuclease, is filled by DNA polymerase ß (WIEBAUER and JIRICNY, 1989; WIEBAUER and JIRICNY, 1990).

Thymine is a natural DNA base; because this thymine DNA glycosylase does not excise single-stranded or matched double stranded substrates, JIRICNY and coworkers proposed that it is activated by a DNA binding protein which recognizes the T·G mismatch (WIEBAUER and JIRICNY, 1990). A 200-kDa T·G mismatch specific DNA binding protein has been identified in nuclear extracts of human HeLa cells (JIRICNY *et al*, 1988).

The presence of a T·G-specific DNA binding protein was not observed in *Drosophila melanogaster* and *Saccharomyces cerevisiae* (JIRICNY *et al*, 1988). The fact that in these two organisms only a negligible amount of methylation occurs, supports the concept that like the VSP repair of *E. coli*, the role of the mammalian G·T-specific correction system is to protect the cells from the potential deleterious effects of the spontaneous hydrolytic deamination of 5-methylcytosine (WIEBAUER and JIRICNY, 1989).

Summary of DNA Mismatch Correction Systems

Base pair mismatches arise continually in the DNA of any organism. In *E. coli*, the fidelity of DNA replication appears to be controlled by two polymerase related enzymatic processes: base selection and the editing mechanism at the

replication fork. However, lesions that escape the proofreading function or that arise spontaneously after replication are eligible for repair by postreplicative mismatch correction systems.

FIGURE 4 illustrates the complex, intertwined network of *E. coli* mismatch repair mechanisms that have been characterized to date. Even before an error results during biosynthesis, *E. coli* has at least one repair system which serves to safeguard DNA from a potent mutagenic substrate. The protein product of *mutT* protects DNA from oxidation damage by hydrolyzing 8-oxodGTP from the cell's nucleotide pool. Cells deficient in *mutT* function are characterized by a large increase of A·T to C·G transversions.

During DNA synthesis, the polymerase itself has an ability to (1) select complementary nucleotides and (2) correct noncomplementary base pairing. The subunits responsible for each process are coded by separate genes. *MutD*$^-$ strains are not only defective in proofreading but also seem to have a deficiency in postreplicative mismatch repair; in fact, the observed increase in mutation rate is actually due to the saturation of the methyl-directed mismatch repair system (SCHAAPER and RADMAN, 1989).

Most biosynthetic errors that escape the proofreading mechanism are corrected by the methyl-directed mismatch repair system; in other words, methyl-directed repair seems to be the primary mechanism for the correction of replication errors. The *dam*-directed system can correct mismatches

that lead to both transitions and most transversions. The *mutY* and VSP repair systems are not affected by GATC methylation and correct specific mismatches after replication. The MutY correction process reduces the frequency of G·C to T·A transversions; VSP repair serves to minimize the mutagenic consequence of cytosine methylation by reducing C·G to T·A transitions.

The product of the *mutT* gene protects the DNA from oxidation damage by 8-oxodGTP by hydrolyzing the potent mutagenic substrate prior to its incorporation in the DNA. 8-oxodGTPs that escape MutT or incorporated guanines that oxidize in the DNA are excised by the product of *mutM*.

The compilation of all the information about characterized mismatch repair systems in *E. coli* reveals that protecting DNA from damage is an important, complex procedure. It involves a kaleidoscope of repair strategies that synchronously work together. Elucidating the mechanisms of the identified repair systems along with the discovery of yet unidentified processes is an extraordinary challenge that has the potential to reveal many unexpected and delightful surprises. The following sections are a description of the research done with the intention to elucidate an identified, yet not fully understood, DNA mismatch correction process: the very short patch repair system.

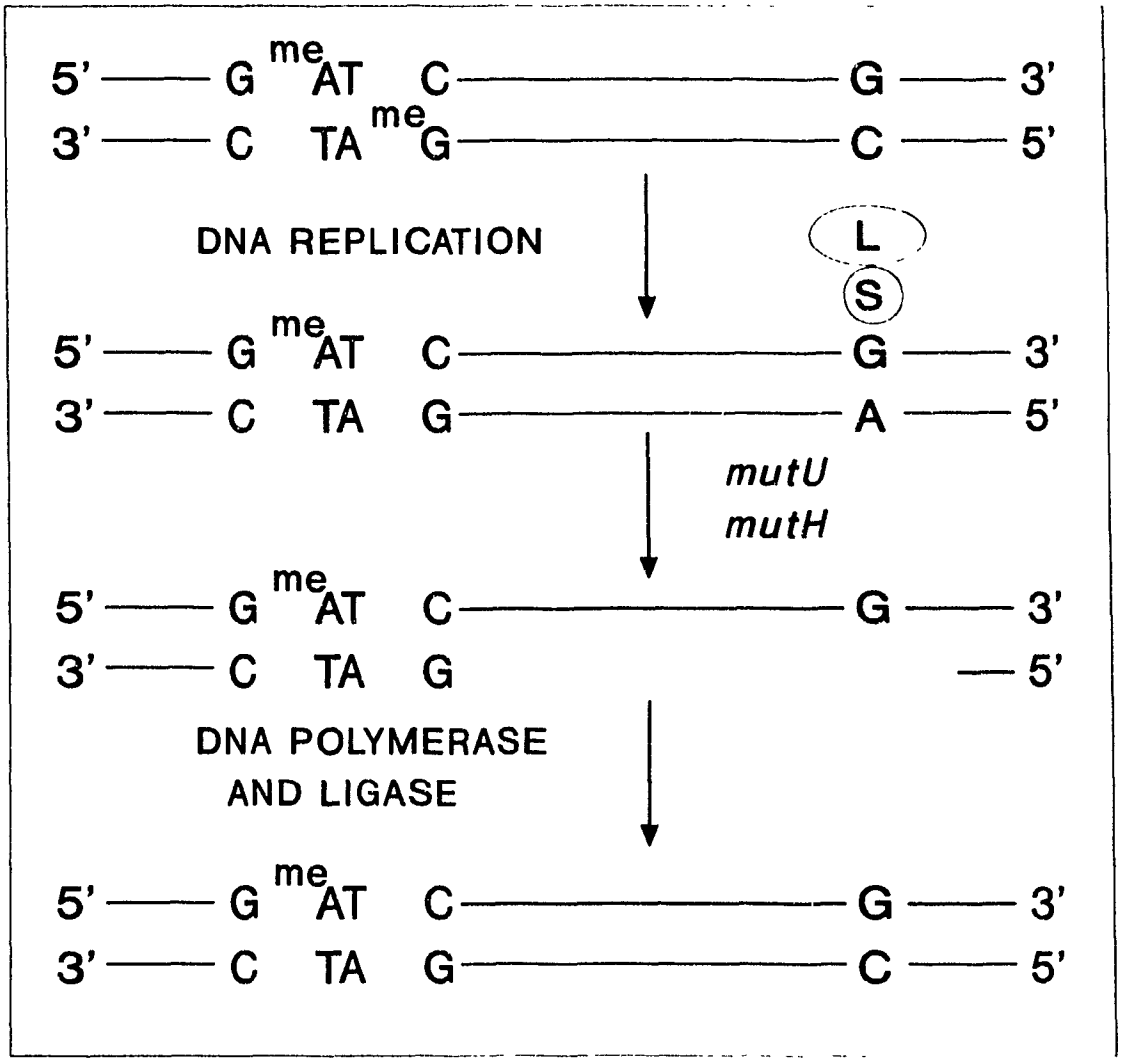

FIGURE 1: METHYL-DIRECTED MISMATCH REPAIR SYSTEM. During DNA replication, the DNA polymerase unwinds both strands which are then used as a template. Adenines at d(GATC) sequences of the parent strands, are methylated. However, in newly synthesized strands, adenines are not methylated. The repair process occurs preferentially on unmethylated strands of hemimethylated heteroduplexes. The product of the *mutS* gene binds to the mismatch. The MutL protein binds to the MutS-heteroduplex complex. The product of *mutH* incises immediately 5' of the dG in the d(GATC) sequence in the unmethylated strand. The DNA helicase II, product of *mutU* unwinds the DNA from the single-stranded break to just beyond the mismatch. The *exonuclease I* hydrolysis of the single-stranded DNA to mononucleotides is enhanced by the adhesion of the SSB protein to the single-stranded DNA. The resulting gap is filled by the DNA polymerase III holoenzyme and the nick is sealed by DNA ligase.

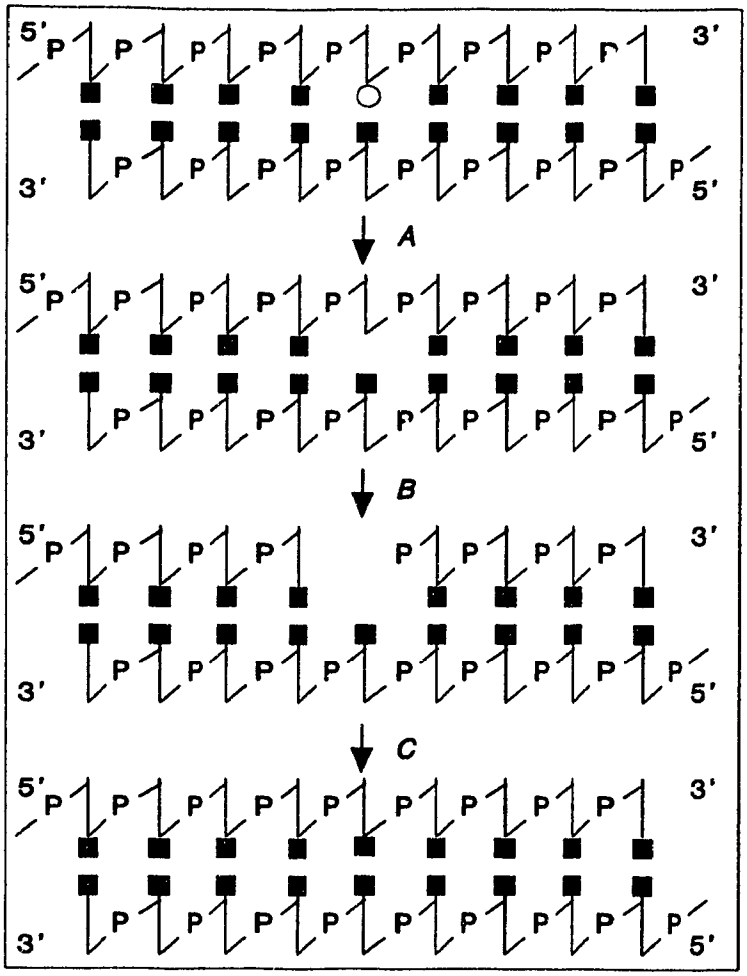

FIGURE 2: MUTY CORRECTION SYSTEM. The *mutY* repair system corrects A·G mispairs to C·G. The mispaired adenine is represented by an open circle. ***A*** A G·A specific glycosylase, product of the *mutY* gene, recognizes the mismatch and hydrolyzes the glycosylic bond which attached the mispaired adenine to the deoxyribose. ***B*** An AP endonuclease cleaves the phosphodiester backbone 5' of the missing base. ***C*** From the 3'-OH group, the DNA polymerase I resynthesizes the complementary fragment. The remaining nick is sealed by DNA ligase.

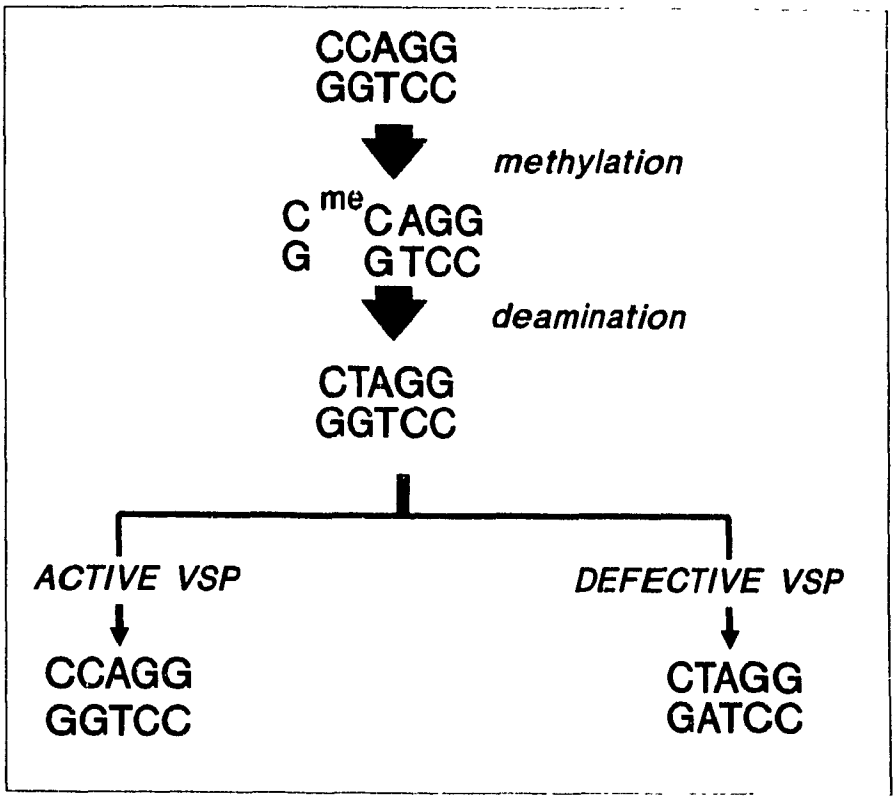

FIGURE 3: VERY SHORT PATCH REPAIR SYSTEM. The product of the deoxycytosine methylase gene, *dcm*, methylates the second C in the sequence CC(A/T)GG to a 5-methylcytosine. Some of these 5-methylcytosines spontaneously deaminate to thymines; forming a T·G mismatch at the second C of the sequence CCAGG. If VSP repair is functional, the T·G mismatch will be repaired and the original C will be restored. However, if VSP repair is not functional, the mismatch will not be corrected and in the next round of replication, C·G to T·A transitions will occur.

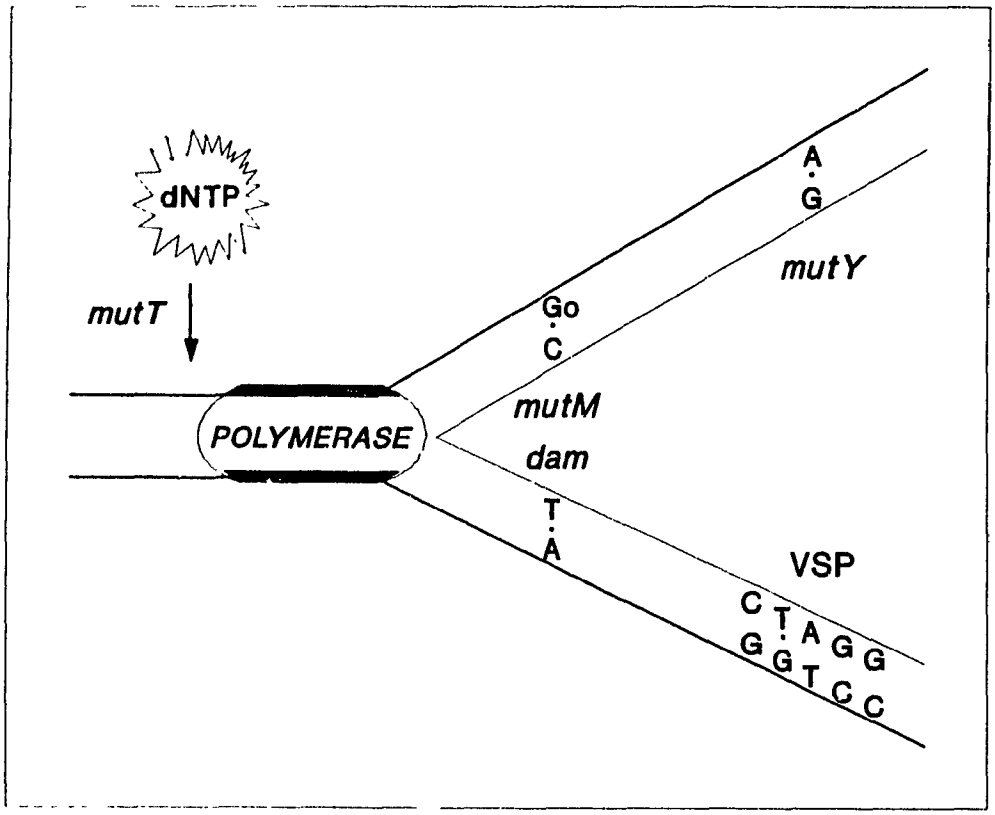

FIGURE 4: SUMMARY OF MISMATCH CORRECTION SYSTEMS. Even before DNA replication, *E. coli* has at least one repair system which serves to safeguard DNA from a potent mutagenic substrate. The protein product of *mutT* protects DNA from oxidation damage by hydrolyzing 8-oxodGTP from the cell's nucleotide pool. Cells deficient in *mutT* function are characterized by a significant increase of A·T to C·G transversions.

During DNA synthesis, the polymerase itself has the ability to (1) discriminate and (2) correct noncomplementary base pairing.

Most biosynthetic errors that escape the proofreading mechanism are corrected by the methyl-directed mismatch repair system; In other words, methyl-directed repair seems to be the primary mechanism for the correction of replication errors. The *dam*-directed system can correct mismatches that lead to both transitions and most transversions. The *mutY* and VSP repair systems are unaffected by GATC methylation and correct specific mismatches after replication. The MutY correction process reduces G·C to T·A transversions; VSP repair serves to minimize the mutagenic consequence of cytosine methylation.

8-oxodGTPs that escape MutT or incorporated guanines that oxidize *in situ* are excised by the product of *mutM*.

MATERIALS AND METHODS

The format of the methods portion of this thesis was designed so as to be as complete and as detailed as possible, hopefully, without being boring. The intent was to allow the reader the option of repeating any or all of the experiments without the need to refer to any other source of information other than the owner's manual for a given piece of equipment the user is not familiar with. In other words, any modification that was made to a procedure has been incorportated in the text. However, should the reader wish to refer to the original protocol, the references have also been noted. Again, the intention was to make the methods section detailed, not repetitive. The following are basic conditions and equipment used for most of the experiments.

Unless otherwise stated, all overnight cultures were made in 2 ml of the given media and were incubated in a 37°C, 250 rpm, shaker incubator.

All work was done using sterile labware and media. Composition of the media used is described in ***APPENDIX A: MEDIA***. Media supplements are described in ***APPENDIX B: MEDIA SUPPLEMENTS***. The composition of the buffers used are listed in ***Appendix C***. The composition of solutions used for rapid plasmid preparation are found in ***APPENDIX D***. For a complete list of the make and model of the commonly used equipment, refer to ***APPENDIX E: COMMONLY USED EQUIPMENT***.

All bacterial strains used are recorded in ***TABLE 1: LIST OF BACTERIAL STRAINS***.

Bacterial strain XA203 is a derivative of strain XAC and contains the plasmid pGFIB (MASSON and MILLER, 1986; NORMANLY *et al.*, 1986) with a cloned synthetic tRNA (for glutamic acid, codon CUA) gene (KLIENA *et al.*, 1990). Strain CC112 is a derivative of XA203 and contains F'CC112 episome (CUPPLES and MILLER, 1988). Strains CC101 to CC106 (CUPPLES and MILLER, 1989) are derivatives of P90C. These seven strains contain an F^- *lacI*$^-$, *lacZ*$^-$, *proAB*$^+$ episome with a different mutation in the *lacZ* gene, at the codon for amino acid 461 of ß-galactosidase. The episomes are referred to as F' CC112 and F' CC101 to F' CC106. Construction of these episomes is described in CUPPLES and MILLER, (1989). The specificity of these episomes is illustrated in ***TABLE 2: STRUCTURE AND FUNCTION OF F' EPISOMES FROM CC101 TO CC106.***

Primers were named by the gene or plasmid from which they were derived followed by the nucleotide number of their 5' base. The nucleotide sequences of all oligonucleotides used are recorded in ***TABLE 3: LIST OF PRIMERS.***

Primers **DCM:-007** and **VSR:2262** were used for the amplification of the *dcm-vsr* genes. The following primers were used for the sequencing of the *vsr* gene from the amplified fragment: **DCM:1619**, **VSR:1806**, **VSR:1951**, and **VSR:2145**. The sequencing of the lambda fragment cloned into pACYC184 was done using oligonucleotides **PACYC:1848** AND **PACYC:1893.**

The methods portion of the thesis is divided into the following five parts:

PART 1: THE SPECIFICITY OF SPR112.

PART 2: MAPPING THE MUTATION.

PART 3: IS THE DEOXYCYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112?

PART 4: DOES VSR COMPLEMENT THE MUTATION?

PART 5: SPR112'S VSR GENE, WILD TYPE OR MUTANT?

PART 6: FINER MAPPING AND CLONING OF MUTG.

PART 1: THE SPECIFICITY OF SPR112

A mutator strain with a high Lac^+ reversion frequency was isolated by C.G. CUPPLES as described by NGHIEM *et al.*, 1988. Cells containing the F'CC112 episome and the glutamic acid inserting suppressor can revert to Lac^+ by suppression of the amber codon at 461 which resulted from a C·G to T·A transition mutation, or by a C·G to T·A transition which would restore the glutamic acid codon at 461.

Transferring F'CC112 episome from SPR112 Lac^+ revertants to S90C.

To determine which base change was indeed happening in SPR112, the F'CC112 episome was isolated from Lac^+ revertants and was transferred to a nonsuppressor background, S90C.

Blue papillae were picked from SPR112 and were streaked onto fresh minimal glucose + 5-bromo-4-chloro-ß-D-galacto-

side (x-gal) + phenyl-ß-D-galactopyranoside (p-gal) + ampicillin. Plates were incubated at 37°C for two days. For each, single blue colonies were purified by restreaking onto fresh media.

To transfer the F'CC112 episome to the nonsuppressor background, Lac^+ revertants were crossed with S90C. The following describes the protocol used for a single cross. For each purified papilla, the method was repeated. A culture of a purified blue colony derived from a Lac^+ SPR112 papillae was made in minimal glucose + ampicillin. The culture was incubated at 37°C until stationary phase. A log phase culture of S90C was made in LB. In a test tube, 200 μl of S90C and 100 μl of Lac^+ revertant cultures were combined. The mixture was incubated at 37°C for 1 hour without aeration. Once the time had elapsed, a loopful was streaked for single colonies onto minimal glucose + x-gal + streptomycin plates. These were incubated at 37°C for two days. The phenotype was observed and recorded.

SPR112 has a high mutation rate. The specificity of these mutations was monitored by curing SPR112 of the F' CC112 episome and introducing six tester episomes, F'CC101 to F'CC106. These six tester episomes detect various mutations at codon 461 of the *lacZ* gene in *E. coli*. For details concerning the structure and function of episomes F' CC101 to F' CC106, refer to ***TABLE 2: STRUCTURE AND FUNCTION OF F' EPISOMES FROM CC101 TO 106.***

Curing SPR112 of the F' CC112 episome

A culture of SPR112 was made in minimal glucose and was incubated until stationary phase. A culture of JM801 was made in minimal glucose + proline + kanamycin and was incubated at 37°C until log phase. In a test tube, 200 µl of SPR112 and 100 µl of JM801 were combined. The mixture was incubated at 37°C for 1 hour without aeration. Once the time had elapsed, a loopful was streaked for single colonies onto minimal glucose + nalidixic acid + arginine + proline + kanamycin plates. These were incubated at 37°C for two days. Colonies were purified on fresh plates which were again incubated at 37°C for two days. To make sure the episome had been lost, colonies were streaked on fresh plates that did not contain any proline; colonies which corresponded to those that did not grow on the proline$^-$ media were used.

Introducing F' CC101 to F' CC106 into SPR112 and CC112

An overnight culture was made of SPR112/JM801 in minimal glucose + nalidixic acid + kanamycin + arginine + proline solution and were incubated until stationary phase. Cultures of CC101 to CC106 were made in minimal glucose solution and were incubated until log phase. SPR112/JM801 was individually crossed with all six F' CC101 to F'CC106 by combining 200 µl of SPR112/JM801 to 100 µl of CC101 to CC106, individually, in separate test tubes. Test tubes were incubated at 37°C, without aeration, for 1 hour. A loopful of each cross was streaked onto minimal glucose + ampicillin

plates.

The same procedure was used to cross CC112 with F' CC101 to F' CC106. 200 μl of stationary phase CC112 culture was individually combined with a 100 μl of log phase CC101 to CC106 culture. These were incubated at 37°C for 1 hour without aeration. Then a loopful of each cross was plated onto minimal glucose + ampicillin plates. Colonies were purified on fresh plates.

The F'CC101 episome has an amber (TAG) codon at 461. In the presence of the glutamic acid inserting suppressor, all cells are Lac^+. To monitor A·T to C·G transitions, SPR112 was cured of the plasmid pGFIB.

Curing SPR112 of the plasmid pGFIB

An overnight culture of SPR112 was made in LB, so as to not select for the plasmid pGFIB. The culture was diluted to 10^{-6} in LB media and a 100 μl aliquot was plated onto LB plates. The plates were incubated overnight at 37°C. The next day, colonies were gridded onto LB plates and were left in a 37°C incubator overnight to grow. When colonies had grown, the plates were replica plated first onto LB plates and then onto LB + ampicillin plates. These plates were incubated at 37°C overnight and for two days respectively. Colonies that did not grow on LB + ampicillin plates were identified and purified. These colonies had lost plasmid pGFIB. The F'CC112 episome was replaced with the F'CC101 episome as described in the previous section.

The number of Lac$^+$ revertants per 10^8 cells

Overnight cultures of SPR112 X CC101 to 106 (SPR101-106) and CC112 X CC101 to CC106 were made in minimal glucose + ampicillin. The following describes the technique used for one culture. The same protocol was used for all overnight cultures. A 100 μl aliquot of the culture was spread onto a minimal lactose + ampicillin plate. A 1 ml portion of the culture was placed in a 1.5 ml microtube. It was centrifuged at full speed in the bench top microfuge for two minutes. The supernatant was aspirated and the pellet was resuspended in 100 μl of minimal lactose solution; the whole suspension was plated onto a minimal lactose + ampicillin plate. The balance of the overnight culture was diluted to 10^{-6} in minimal glucose and a 100 μl aliquot was spread onto an LB plate. All plates were incubated at 37°C for two days. The number of colonies on each plate was counted. The Lac$^+$ reversion frequency was calculated for the 100 μl and the 1 ml aliquots of undiluted culture. Only the data obtained from cultures which gave more than one hundred colonies on LB plates were used.

PART 2: MAPPING THE MUTATION

The mutation in SPR112 was mapped by P1 transduction. The following strains, a subset of the SINGER mapping kit (SINGER *et al.*, 1989), were used: 12068, 12156, 18451, 12099, 12179, 18467. The following describes the protocol

used for one mapping strain. The technique was applied to all the strains used.

Making a lysogen/ lysate

A P1cm lysate was streaked in a straight line in the middle of a chloramphenicol plate. Before being absorbed into the plate, a heavy inoculum of the mapping strain was cross streaked, zigzagging across the P1cm lysate. The plate was incubated at 28°C - 31°C for 2 days. Colonies were purified on chloramphenicol plates which again were incubated at 28 - 31°C for a couple of days. To ensure the quality of the lysogen, a duplicate plate was made, (using the same colonies) but was incubated at 42°C. Colonies should grow at 28°C and not at 42°C due to the strain's temperature sensitivity.

Overnight cultures of the P1cm/mapping strain lysogens were made by inoculating 2 ml of superbroth + chloramphenicol and incubating at 28°C - 31°C in a shaker water bath. The following day, the cultures were subcultured by inoculating 2 ml of superbroth + chloramphenicol with 100 µl of the overnight culture. The cultures were grown at 28°C to 31°C until a distinct cloudiness was observed in the tube (log phase). To induce lysis, the cultures were transfered to a 42°C shaker waterbath for half an hour after which they were transfered to the 37°C shaker incubator until lysis occured; usually, a sufficient decrease in turbidity was observed after a two hour incubation period. The cultures

were transfered to 15 ml polypropylene conical centrifuge tubes. To each tube, one ml of chloroform was added. The tubes were vortexed and then centrifuged for 2 minutes at room temperature. Top layers were transfered to separate glass screw cap test tubes which contained a few drops of chloroform. Tubes were stored at 4°C.

Transduction

An overnight culture of the recipient strain was made in 2 ml of minimal glucose + ampicillin media. The following day, the cells were centrifuged and the supernatant was aspirated. The cells were resuspended in one volume of MC buffer (100 mM calcium chloride and 1 mM magnesium sulphate), and incubated in a 37°C shaker incubator for 20 minutes. Meanwhile, a fraction of the phage was diluted to 10^{-1} and 10^{-2} in superbroth. Five tubes were set up as follows: the first four contained 0.1 mls of SPR112 culture. 0.1 ml of undiluted phage was added to tubes #2 and #5. 0.1 mls of phage diluted to 10^{-1} and 10^{-2} was added to tubes #3 and #4, respectively. The tubes were incubated at 37°C with aeration for 30 minutes. 0.1 ml of 25% sodium citrate solution and 1.0 ml of LB liquid media was added to all tubes. These were outgrown by incubating in a 37°C, 250 rpm, shaker incubator for 1 hour. 100 µl of each sample was individually plated on the following selective plates: minimal glucose supplemented with x-gal, p-gal, ampicillin, and tetracycline. The plates were incubated at 37°C for 1

week. The number of colonies with less than three papillae and the total number of colonies were recorded. Colonies with less than three papillae were streaked for single colonies on fresh selective plates. Again, the ratio of colonies with less than three papillae to the total number of colonies was calculated and recorded.

PART 3: IS THE DEOXYCYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112?

The purpose of this experiment was to determine if cytosines are methylated in SPR112. The following strains were used: CC112, RP4182, and SPR112. CC112 and RP4182 were used as controls. CC112 has a wild type deoxycytosine methylase gene, *dcm*; RP4182 is dcm^- due to a chromosomal deletion at 43 minutes. Chromosomal DNA was isolated from the three strains and was digested with the restriction enzyme *Eco*RII.

The isolation of CC112, RP4182, and SPR112 chromosomal DNA

The method employed was based on the technique reported by SILHAVY *et al.* (1984) and the modifications reported by WHORISKEY *et al.*(1987).

DNA extraction from bacterial cells

An overnight culture of each strain was made in 2 ml of LB. Three 250 ml Erlenmeyer flasks containing 100 ml of LB

were inoculated individually with 100 μl of one of the three overnight cultures. The flasks were then incubated in a shaker-incubator at 37°C, 250 rpm, until late log/early stationary phase. Cultures were decanted into 50 ml Oak Ridge centrifuge tubes and were centrifuged at room temperature for twenty minutes at 2000 g. The supernatant was aspirated and the pellet was resuspended in 5 ml of 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA. Each suspension was then transferred to separate 15 ml polypropylene conical centrifuge tubes. The suspensions were then placed in a -20°C freezer until they were completely frozen. 0.5 ml of freshly made lysozyme solution (10 mg/ml in 0.25 M Tris-HCl, pH 8.0) was added to each frozen suspension, and with gentle mixing, these were thawed in a room temperature water bath. As soon as the contents had thawed, the tubes were placed on ice for 45 minutes. One ml of STEP solution (5% SDS, 0.05 M Tris-HCl (pH 8.0), 0.4 M EDTA (pH 8.0), and 1 mg/ml proteinase K) was added to the samples. After thorough mixing, the samples were heated at 55°C for 90 minutes with occasional, gentle mixing. Six ml of Tris buffered phenol (pH 8.0) was added to each sample. For about five minutes, the samples were mixed gently to emulsify. To separate the aqueous and organic layers, the samples were centrifuged at 1000g for 15 minutes. For each sample, the aqueous top layer, which contains the DNA, was transfered to a clean 15 ml polypropylene conical centrifuge tube. Care was taken not to include the material at the interface. The phenol extraction

was repeated two more times; however, for these two steps, a 1:1 ratio of phenol to chloroform was used. The aqueous top layer was transfered to a clean 15 ml polypropylene centrifuge tube. To precipitate the DNA, 0.1 volume of 3 M sodium acetate and 2.5 volumes of cold ethanol were added. Mixing was done by gently inverting the tubes. At this point, the DNA and RNA precipitated. The precipitate was carefully spooled out by coiling it around the surface of a sterile glass Pasteur pipette. Excess alcohol was removed by gently rotating the precipitate against the side of the tube. The precipitate was transfered to a clean 15 ml polypropylene conical centrifuge tube which contained 5 ml of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 200 μg/ml RNAase. The samples were incubated in a 37°C shaker water bath for two hours. Before pursuing any further, it was essential to have the DNA completely dissolved. If the DNA was not completely dissolved, more buffer was added. An equal volume of chloroform was added and the samples were inverted until an emulsion had formed. To separate the layers, the samples were centrifuged at 1000g for 15 minutes. The aqueous top layer was transfered to a clean 15 ml centrifuge tube. 0.1 volume of 3 M sodium acetate, followed by two volumes of 95% ethanol were added; samples were mixed by inversion. DNA precipitated as long threadlike structures. The DNA was spooled out with a sterilized Pasteur pipette. Excess alcohol was removed by gently rotating the precipitate against the side of the tube. The DNA was completely dissolved in 2 ml of 50

mM Tris-HCl, pH7.5, and 1 mM EDTA. The concentration of DNA was determined by measuring the absorbance at 260nm. The final concentration of CC112, RP4182, and SPR112 chromosomal DNA was 266μg/ml, 57μg/ml, and 145μg/ml respectively.

Chromosomal DNA Digested with EcoRII

1330 ng, 570 ng, and 725 ng respectively of CC112, RP4182, and SPR112 chromosomal DNA were introduced into 1.5 ml microtubes. To each sample, 2 units of *Eco*RII restriction enzyme (BRL) and 1 μl of the 10X concentrate assay buffer (supplied with the restriction enzyme) was added. To each sample, sterile deionized water was added to a final volume of 12 μl. Samples were centrifuged briefly in a room temperature benchtop microfuge, and were incubated in a 37°C water bath for 6 hours. Samples were again centrifuged. Two μl of gel loading dye was added to each sample and these were electrophoresed on a 1% agarose gel accompanied by the same quantity of undigested chromosomal DNA from the three strains.

PART 4: DOES VSR COMPLEMENT THE MUTATION?

To determine if the mutator phenotype of SPR112 was due to a mutation in *vsr*, a complementation experiment was performed. SPR112 was transformed with pDCM28. Plasmid pDCM28 contains only the promoter region of *dcm* and the whole *vsr* gene cloned into the *Sph*I-*Bam*HI site of plasmid

pACYC184.

Making SPR112 competent

This method is based on the variation of the method of COHEN (COHEN *et al.*, 1972) reported by SAMBROOK (SAMBROOK *et.al.*, 1982). An overnight culture of SPR112 was made in 2 ml of minimal glucose + ampicillin. The following day, 40.0 ml of minimal glucose + ampicillin in a 250 ml Erlenmeyer flask was inoculated with 0.3 ml of the SPR112 culture. The flask was incubated in a 37°C shaker incubator until the OD_{550} was between 0.2 and 0.25. To stop cell growth, the flask was put on ice for ten minutes. The contents were then poured into a 50.0 ml Oak Ridge centrifuge tube which was centrifuged at 4°C for 5 minutes at 5000 rpm. Once the supernatant was aspirated, the cells were gently resuspended in half the original volume cold 50 mM calcium chloride and were left on ice for half an hour. Once the time had elapsed, the cells were again centrifuged at 4°C for 5 minutes at 5000 rpm. The supernatant was aspirated and the cells were gently resuspended in one tenth the original volume (4.0 ml) cold 50 mM calcium chloride.

The transformation of SPR112 with pDCM28:

In a 1.5 ml microtube, 200 μl of competent SPR112 cells were gently mixed with 2 μl of pDCM28 and was left on ice for thirty minutes. The tube was then transfered for half an hour to a 42°C water bath. Once the time had

elapsed, the tube was put back on ice where 800 μl of LB media was added. Cells were incubated in a 37°C water bath for one hour (ie. for chloramphenicol expression). Samples were centrifuged at full speed for 1 minute in the benchtop microfuge. The supernatant was aspirated and the pellet was resuspended in 200 μl of LB media. Half the sample, 100 μl, was spread on the following selective plates: minimal glucose + ampicillin + chloramphenicol. Plates were incubated at 37°C for two days.

The number of Lac$^+$ revertants per 10^8 cells

Overnight cultures of hundreds of transformants were made in 2 ml of minimal glucose + ampicillin + chloramphenicol. The following describes the technique used for one culture. The same protocol was used for all transformants of which an overnight culture was made. A 100 μl aliquot of the culture was spread onto a minimal lactose + ampicillin + chloramphenicol plate. A 1 ml portion of the culture was placed in a 1.5 ml microtube. It was centrifuged at full speed in the bench top microfuge for two minutes. The supernatant was aspirated and the pellet was resuspended in 100 μl of minimal glucose solution; the whole suspension was plated onto a minimal lactose + ampicillin + chloramphenicol plate. The balance of the overnight culture was diluted to 10^{-6} in minimal glucose and a 100 μl aliquot was spread onto an LB plate. All plates were incubated at 37°C for two days. The number of colonies in each plate was counted. The Lac$^+$

reversion frequency was calculated for the 100 µl and the 1 ml aliquots of undiluted culture. Data obtained from LB plates that had at least a viability of 10^9 colonies per ml was used.

PART 5: SPR112'S VSR GENE: WILD TYPE OR MUTANT?

To determine if the *vsr* gene from CC112 and SPR112 was wild type, the polymerase chain reaction was used to amplify *dcm* and *vsr* from the two strains and the *vsr* genes were sequenced. For this experiment, the chromosomal DNA was the same as that used in ***PART 2: IS THE CYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112***; please refer to that section for the protocol.

Amplification of dcm-vsr from CC112 and SPR112 using the polymerase chain reaction

For the amplification step, the Bellco Biotechnology DNA PACER was used. Primers **VSR:2262** and **DCM:-007**, courtesy of A. S. Bhagwat, were used for the amplification of the *dcm-vsr* genes from CC112 and SPR112. For the nucleotide sequence of these primers, refer to ***TABLE 3: LIST OF PRIMERS***. It should be noted that *Bam*HI and *Sph*I cut sites have been integrated in the primers **VSR:2262** and **DCM:-007** respectively.

Duplicates of each of the following were made.

In microtube A: 7 μl of CC112 chromosomal DNA (266 ng/μl)

1 μl of primer **VSR:2262** (386 ng/μl)

1 μl of primer **DCM:-007** (368 ng/μl)

10 μl of 2 mM dNTP

70 μl sterile water

In microtube S: 7 μl of SPR112 chromosomal DNA (145 ng/μl)

1 μl of primer **VSR:2262** (386 ng/μl)

1 μl of primer **DCM:-007** (368 ng/μl)

10 μl of 2 mM dNTP

70 μl sterile water

The reaction mixtures were heated to 96°C for 3 minutes. Then, they were cooled to 75°C for 20 seconds during which 10 μl of 10X *Taq* polymerase buffer, supplied with the enzyme, and 1 μl of *Taq* polymerase (Bio/Can) was added to each reaction tube. For 35 cycles, the following PCR parameters were used:

(1) denaturing: 95°C for 20 seconds; ramp 3.0

(2) annealing: 60°C for 1 minute; ramp 3.0

(3) extension: 72°C for 45 seconds; ramp 3.0

To verify if amplification of the *dcm-vsr* genes was successful, a 10 μl sample of the PCR product was electrophoresed, at 125 volts, on a 1% agarose gel.

With the intention of sequencing *vsr* from single stranded *dcm-vsr*, the double stranded *dcm-vsr* was extracted with phenol:chloroform and precipitated with ethanol to remove excess primer.

Cleaning the double stranded dcm-vsr for use in asymmetrical PCR:

The PCR products of two successful PCR reactions were combined in a 1.5 ml microtube. To 180 µl of double stranded DNA, 100 µl phenol and 100 µl chloroform were added. The mixture was vortexed briefly and centrifuged in the benchtop centrifuge at room temperature for two minutes. The top layer was transferred to a new microtube and 60 µl of 10 M ammonium acetate was added. The sample was left at room temperature for two minutes. 800 µl of 95% ethanol was added. The sample was incubated at -20°C for three minutes and was centrifuged at room temperature for thirty minutes. With a micropipet, the supernatant was carefully removed and the pellet was dried in the lyophilizer. The pellet was resuspended in 100 µl of sterile distilled water.

To verify that the amplified DNA was not lost in the cleaning, 2 µl, 5 µl, and 10 µl samples of the template were electrophoresed on a 1% agarose gel. Usually, clear bands could be observed with the 2 µl samples and the intensity increased as the volume of the sample used increased.

Using asymmetrical PCR to produce ss DNA for sequencing

To sequence *vsr* from CC112 and SPR112, single-stranded *dcm-vsr* was produced by asymmetrical PCR. Reactions were carried out at the parameters stated below in 0.5 ml microtubes containing the following:

microtube A-1:

2 μl ds CC112 *dcm-vsr*

2 μl primer **VSR:2262**

2.5 μl 2 mM dNTP

83 μl H_2O

10 μl 10X *Taq* buffer

1 μl *Taq* polymerase

microtube A-2

2 μl ds CC112 *dcm-vsr*

2 μl primer **DCM:-007**

2.5 μl 2 mM dNTP

83 μl H_2O

10 μl 10X *Taq* buffer

1 μl *Taq* polymerase

microtube G-1:

2 μl ds SPR112 *dcm-vsr*

2 μl primer **VSR:2262**

2.5 μl 2 mM dNTP

83 μl H_2O

10 μl 10X *Taq* buffer

1 μl *Taq* polymerase

microtube G-2:

2 μl ds SPR112 *dcm-vsr*

2 μl primer **DCM:-007**

2.5 μl 2 mM dNTP

83 μl H_2O

10 μl 10X *Taq* buffer

1 μl *Taq* polymerase

The 100μl reaction mixture was heated to 75°C for two minutes (note: as opposed to the double standed symmetrical amplification, the *Taq* polymerase and buffer were already in the tube). The following PCR parameters were used:

(1) denaturing: 95°C for 20 seconds; ramp 3.0

(2) annealing: 62°C for 1 minute; ramp 3.0

(3) extension: 72°C for 45 seconds; ramp 3.0

cycles: 30.

PURIFICATION OF THE ASYMMETRICAL PCR PRODUCT FOR SEQUENCING

The following protocol was based on the method described in *PCR Protocols: A Guide to Methods and Applications* (INNIS *et al.*,1990). 100 μl of 4 M ammonium acetate

(NH_4OAc) was added to 100 μl of the asymmetrical PCR mixture. 200 μl of 2-propanol was then added. Samples were well mixed and incubated at room temperature for ten minutes. DNA was collected by centrifugation at full speed in a benchtop microcentrifuge for ten minutes. Using a micropipet, the supernatant was carefully removed and discarded. The pellet was rinsed with 500 μl of 70% ethanol. Again, samples were centrifuged in a benchtop microfuge at room temperature for ten minutes. The supernatant was carefully removed and the pellet was dried in a Speed Vac. Once dried, the DNA was dissolved in 10 μl TE buffer. Fifty to 100% of the sample was used as template for sequencing. The Pharmacia ^{T7}SEQUENCING ^{TM} KIT (ie: dideoxy sequencing reactions using T7 polymerase) was used. Primers **DCM:1619** and oligo **VSR:1806** were used to sequence single stranded *vsr* amplified with primer **VSR:2262**; Primers **VSR:2145** and oligo **VSR:1951** were used to sequence *vsr* from the single stranded *dcm-vsr* template made with oligo **DCM:-007**.

PART 6: THE FINER MAPPING AND CLONING OF MUTG

Yuji KOHARA (KOHARA *et al.*, 1987) has cloned the entire *E. coli* genome in approximately 10-Kb fragments into lambda phage. The location of each cloned fragment on the *E. coli* genome is known and the restriction map of each piece has been determined. Knowing that the mutator locus maps at 43 minutes, KOHARA fragments which spanned the 43 minute region

of the *E.coli* chromosome were tested for their ability to complement the mutator phenotype. KOHARA lambda phage do not contain the genes coding for lysogeny, it is a lytic phage which means it lysis cells. This complicated the situation because complementation in SPR112 can only be monitored by an observable phenotype (ie: a decrease in papillation) in living cells. The DNA from lambda phage was subcloned into plasmid pACYC184, introduced into SPR112, and the ratio of papillator to non-papillator was determined.

Subcloning into pACYC184 was done by isolating lambda DNA and partially digesting it with the restriction enzyme *Sau*3A. *Sau*3A recognizes the four nucleotide sequence GATC, which occurs relatively frequently. The plasmid pACYC184 was cut with *Bam*HI. *Bam*HI is a 6 cutter - it recognizes the sequence GGATCC in the tetracycline gene of pACYC184; it should be noted that the *Sau*3A GATC cut site is included in the *Bam*HI restriction enzyme cut site; *Sau*3A - *Bam*HI digestions create compatible single-stranded ends.

Partially digested DNA was ligated into the *Bam*HI site of pACYC184. It should be noted that each *Bam*HI site has a one in four chance of being restored, therefore, one can not cut the plasmid with *Bam*HI and isolate the cloned fragment.

***Isolation of lambda DNA*:**

The protocol is divided into 8 parts. Parts ***(a)***, ***(b)***, and ***(c)*** were based on the technique described in DAVIS (DAVIS *et al.*, 1980); parts ***(d)*** through ***(h)*** are based on

SAMBROOK'S protocol (SAMBROOK *et al.*, 1982).

(a) Preparing LE392 for lambda phage infection

In a 250 ml Erlenmeyer flask, 100 ml of LB + 0.2% maltose (maltose was used to induce maltose transporters which are lambda receptors in the host LE392) was inoculated with one fresh colony of LE392. The flask was incubated in a 37°C shaker incubator until the OD_{600} was 1.0. The cells were then decanted into 500 ml Oak Ridge centrifuge bottles and centrifuged at 8000 rpm for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 50 ml (half the original volume) of 10 mM $MgSO_4$ and stored at 4°C.

KOHARA phage #343 to 351, inclusive were used. The following describes the protocol used for one phage; the same procedure was repeated for all KOHARA phage used.

The phage was diluted to 10^{-2}, 10^{-4}, and 10^{-6} in LB media. In a test tube, 10 μl of diluted phage was added to 200 μl of LE392 cells; the mixture was incubated at 37°C for 10 minutes without aeration. Three ml of top agar, kept warm at 55°C, was added to the tube which was then vortexed and poured onto LB plates. Once cooled, the plates were incubated at 37°C overnight.

(b) Propagate phage

Plates with confluent lysis and smallest dilution were used. Three ml of superbroth were added to the plates. Plates were rotated at low speed for two hours. Once the

time had elapsed, using a 5 ml pipette, the superbroth containing suspended phage was removed and put into separate 15 ml conical polypropylene centrifuge tubes. Chloroform, about one third of the phage suspension volume, was added to the tube. Once vortexed, the tube was centrifuged at full speed for two minutes. The supernatant was transfered to a new tube. The step was repeated. The supernatant was transfered to a glass screw cap test tube and was stored at 4°C.

(c) Titer phage

The phage was diluted to 10^{-6} in superbroth. 100 μl of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were separately added to 100 μl of LE392 that had been prepared for phage infection as described in section ***(a) Prepare LE392 for lambda phage infection*** of this ***PART 6: THE FINER MAPPING AND CLONING OF MUTG***. The mixture was incubated at 37°C for 10 minutes without aeration. Three ml of top agar, kept liquid at 55°C, was added to each tube, was immediately vortexed and poured onto LB plates. Once solidified, the plates were inverted and incubated at 37°C overnight. The following day the titer of the phage was determined.

(d) Infection at high multiplicity

An overnight culture of LE392 was made by inoculating 2 ml of LB + maltose.

In a 250 ml Erlenmeyer flask, 100 ml of NZCYM media (for composition, refer to ***APPENDIX A: MEDIA***) prewarmed to

37°C, was inoculated with 200 µl of LE392 overnight. The flask was left in a 300 rpm, 37°C shaker-incubator until the OD_{600} of the cultures was 0.5. Each flask was inoculated with 10^8 to 10^{10} pfu (plaque forming units) of lambda. The flasks were incubated at 37°C and 300 rpm until lysis occurred (about three to five hours). Two ml of chloroform was then added to each flask and shaking was resumed at 37°C, for another 10 minutes.

(e) Purification of bacteriophage lambda:

Once cooled to room temperature, pancreatic DNAase and RNAase, each to a final concentration of 1 µg/ml, were added to each flask and left at room temperature for 30 minutes. By swirling, solid NaCl (final concentration of 1 M) was dissolved in each flask and left on ice for 1 hour. Debris was removed by transfering the contents of the flask to a 500 ml Oak Ridge centrifuge bottle and centrifuging at 11,000g. Supernatant was transfered to a clean, sterile, 250 ml Erlenmeyer flask containing a magnetic stirbar. Solid polyethylene glycol (PEG), to a final concentration of 10% W/V was dissolved by slow stirring on a magnetic stirer at room temperature. To form a precipitate, the bacteriophage particles were incubated in ice water for 1 hour. Bacteriophage particles were recovered by centrifugation at 11 000g for 10 minutes at 4°C. The supernatant was discarded. Further, the bottle was tilted for 5 minutes in a position which allowed remaining fluid to drain away from pellet. The

remaining fluid was removed by using a Pasteur pipette. The bacteriophage pellet was resuspended using a wide-bore pipette equipped with a rubber bulb in SM media (1.6 ml per 100 ml of supernatant prior to the addition of PEG).

Remaining cell debris and polyethylene glycol was extracted from the bacteriophage suspension by adding an equal volume of chloroform and vortexing for 30 seconds. The aqueous and organic phases were separated by centrifugation at 3 000g for 15 minutes at 4°C. The aqueous phase, which contained the bacteriophage particles, was recovered; the organic phase was discarded.

(f) Pelleting bacteriophage lambda:

Bacteriophage particles were isolated by spinning in an ultracentrifuge at 25 000 rpm for 2 hours at 4°C using a Beckman SW28 rotor. The supernatant was discarded. A glassy pellet of bacteriophage particles was visible at the bottom of the tube. The pellet was resuspended in 1.5 ml of SM solution (for composition, refer to ***APPENDIX C***) by leaving the tube overnight at 4°C. The next day, the solution was gently pipetted up and down to ensure that all bacteriophage particles had been resuspended.

(g) Extraction of bacteriophage lambda DNA

Proteinase K, to a final concentration of 50 µg/ml was added to each tube. SDS, from a 10% w/v solution was added so as to make a final concentration of 0.5%. The tube was

mixed by inverting it a few times and was incubated at 56°C for 1 hour. The digestion mixture was cooled to room temperature and transfered to a centrifuge tube to which an equal volume of phenol, equilibrated with 50 mM Tris (pH 8.0), was added. The tube was mixed by inversion until a complete emulsion had formed. The phases were separated by centrifugation at 3 000g for 5 minutes at room temperature. A wide-bore pipette was used to transfer the aqueous phase to a clean tube. The procedure was repeated two more times first using a 50:50 mixture of equilibrated phenol and chloroform and finally with chloroform only.

(h) Isolation of bacteriophage DNA via ethanol precipitation

From a 3 M stock solution (pH 7.0), sodium acetate was added to a final concentration of 0.3 M. The tubes were gently vortexed. To each tube, two volumes of ethanol were added; tubes were mixed and were left at room temperature for 30 minutes.

Bacteriophage lambda DNA was seen as a threadlike precipate which was recovered from the solution by coiling it onto the surface of a sterile Pasteur pipette. The DNA was transfered to a microfuge tube containing 1 ml of 70% ethanol for rinsing. The DNA was recovered by centrifugation at 12 000g for 2 minutes at 4°C in a benchtop microfuge. The supernatant was discarded and the remaining pellet of DNA was dried at room temperature. The DNA was resuspended in TE.

Sau3A partial digest of lambda DNA:

Quadruplicates of each sample were made. To 5 μl of the isolated lambda DNA, 2 μl of 10X Buffer supplied with the restriction enzyme, 1.25 units of *Sau*3A restriction enzyme (Bio/Can), and sterile deionized distilled water to a final 10 μl were added. The samples were incubated in a 37°C water bath for the following time periods: the first: 10 minutes, the second: 15 min., the third: 20 minutes, and the fourth: 25 min. Once the incubation time elapsed, 1 μl of the sample was added to a microtube containing 2 μl of loading dye and 7 μl of sterile distilled water. Samples were electrophoresed on a 1% agarose gel at 110 volts. The desired incubation time was the one which provided the largest range of fragment sizes. The ten minute incubation time was chosen. The experiment was repeated in 0.5 ml microtubes and this time, the Bellco DNA Pacer was used. Samples, topped with mineral oil, were incubated at 37°C for ten minutes only. The *Sau*3A restriction enzyme was inactivated by heating the sample to 85°C for thirty minutes. To make sure the samples were at the bottom of the microtubes, they were centrifuged briefly at full speed in the benchtop microfuge.

Cutting pACYC184 with BamHI:

This procedure was also carried out in 0.5 ml microtubes. To 10 μl of the pACYC184 plasmid prep, 2 μl of 10X buffer supplied with the restriction enzyme, 2 μl of RNA'se, 20 units of *Bam*HI restriction enzyme (Pharmacia), and 2 μl

of water were added; sample was topped with 50 μl of mineral oil. Using the Bellco DNA Pacer, the sample was incubated at 37°C for one hour. Then, to inactivate the restriction enzyme, *Bam*HI, the sample was heated at 85°C for 30 minutes. To see if the digest was successful, 2 μl of sample (with 2 μl loading dye and 6 μl of water) were electrophoresed on a 1% agarose gel. Samples that were completely digested were used.

Cloning lambda Sau3A partials into pACYC184

The following were put in a 1.5 ml microtube: 2 μl of *Bam*HI digested pACYC184, 4 μl of *Sau*3A digested lambda DNA, 12 units of T4 DNA ligase (Bio/Can), 1μl 10X ligase buffer supplied with enzyme, and water so as to make a final 10 μl volume. The mixture was left at room temperature overnight.

Transforming SPR112 with (pACYC184 + lambda inserts)

SPR112 was made competent and was transformed with plasmid (pACYC184 + lambda inserts). For protocol, refer to ***PART 4: DOES VSR COMPLEMENT MUTG?*** sections ***Making SPR112 competent*** and ***The transformation of SPR112 with pDCM28***. Transformants were plated onto minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates. Plates were incubated in a 37°C incubator for one week. The 544 colonies which exhibited a low papillation phenotype (ie: had less than three papillae) were selected.

Isolation of SPR112 containing (pACYC184 + lambda inserts)

The following test was done to see if the pACYC184 plasmid introduced into SPR112 which exhibited less than three papillae had a lambda DNA fragment cloned into the *Bam*HI site of its tetracycline gene. The colonies with less than three papillae were gridded onto minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates and were incubated for two days at 37°C. Plates were then replica plated first onto a fresh minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plate and then onto a minimal glucose + ampicillin + tetracycline plate. Plates were incubated in a 37°C incubator for one week and for two days, respectively. Only 197 of the 544 colonies were ampicillin resistant, chloramphenicol resistant, and tetracycline sensitive. These were identified. After the one week incubation of the minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates, only six of the 197 colonies retained their "less than three papillae" phenotype; these were designated as SPR112 + (pACYC184 + lambda) #1 to #6. However, when these strains were restreaked onto fresh minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates, SPR112 + (pACYC184 + lambda) #4 and #5 papillated. These were no longer used.

The following approach was used to determine if (pACYC184 + lambda #1, #2, #3, and #6) did have a fragment cloned into its *Bam*HI site. The plasmid was isolated, digested with various restriction enzymes, and electrophoresed

on a 1% agarose gel. However, the situation was complicated by the presence of a second plasmid in SPR112: pGFIB. The problem was overcome as described in the next section.

Rapid plasmid preparation

The following protocol was based on the methods of BIRNBOIM and DOLY (1979) and ISH-HOROWICZ and BURKE (1981). It describes the procedure used to isolate the pACYC184 derived plasmid from one of the two colonies which exhibits a low papillator phenotype and tetracycline sensitivity. An overnight culture was made by inoculating 2 ml of LB + chloramphenicol; no ampicillin was used so as to not select for the plasmid pGFIB. The next day, the culture was transfered to a 1.5 ml microtube and was centrifuged in the benchtop microfuge at full speed for 1 minute. The supernatant was aspirated and the cells were resuspended in 100 µl of solution I (for composition, refer to ***APPENDIX D***) and left at room temperature for 5 minutes. 200 µl of solution II (for composition, refer to ***APPENDIX D***) was added, the tube was inverted 10 times to mix, and then left on ice for 5 minutes. 150 µl of solution III (for composition, refer to ***APPENDIX D***) was added; the contents were mixed by inverting the microtube ten times. Samples were left on ice for 5 minutes. Cells were then centrifuged for 1 minute at full speed in a benchtop microfuge. The supernatant was transfered to a new 0.5 ml microfuge where 400 µl of Tris buffered phenol was added. The sample was briefly vortexed. The

microtube was centrifuged in a benchtop microfuge at full speed for 2 minutes. The top layer was transfered to a new 0.5 ml microtube. The procedure was repeated two more times; first with 400 µl of a 1:1 ratio of phenol: chloroform and second with 400 µl of chloroform only. The top layer was transfered to a new 0.5 ml microtube and 800 µl of 95% ethanol was added; the tube was mixed gently and was left at room temperature for 1 minute. The tube was centrifuged in a benchtop microfuge at full speed for 3 minutes. The supernatant was aspirated and the pellet was dried in a lyophilizer. The dried pellet was resuspended in 30µl of sterile distilled water and stored at -20°C. Competent cells of S90C were transformed with the pGFIB/(pACYC184 + lambda) mixture (for protocol, refer to ***PART 4: DOES VSR COMPLEMENT MUTG***, sections ***Making SPR112 competent*** and ***The transformation of SPR112 with pDCM28***). Transformants were spread onto LB + chloramphenicol plates. Plates were incubated at 37°C overnight. The following day, 50 transformants were gridded onto fresh LB + chloramphenicol plates. These were incubated overnight at 37°C. Plates were replica plated onto LB + ampicillin plates. These plates were incubated overnight at 37°C. Colonies which were chloramphenicol resistant and ampicillin sensitive contained only (pACYC184 + lambda) plasmid and not pGFIB. These colonies were streaked onto fresh LB + chloramphenicol plates and were incubated overnight in a 37°C incubator.

Determining the presence of a lambda insert in pACYC184

A plasmid preparation of (pACYC184 + lambda) #1, #2, #3, and #6 was made using the ***rapid plasmid preparation*** described earlier in this section. To determine if the plasmid had an insert, it was digested with *Hin*dIII and *Sph*I. In a 1.5 ml microtube, 4 μl of the plasmid preparation, 1 μl of *Hin*dIII, 1 μl of *Sph*I, 1 μl of the 10X buffer supplied with the *Hin*dIII restriction enzyme, 1 μl of RNAase, and 2 μl of water were combined. The mixture was incubated in a 37°C water bath for at least 1 hour. To collect the contents at the bottom of the microfuge, the samples were microfuged briefly at room temperature. Then, 2 μl of loading dye was added to each microtube and the samples were electrophoresed on a 1% agarose gel. Of the four plasmid preparations, only (pACYC184 + lambda) #1 and #6 had inserts.

Removing plasmid (pACYC184 + lambda) #1 and #6 from SPR112

Overnight cultures of SPR112 + (pACYC184 + lambda) #1 and #6 were made in minimal glucose + ampicillin solution; to favor the loss of (pACYC184 + lambda) plasmid, no chloramphenicol was included. The overnight culture was diluted to 10^{-6} and a 100 μl aliquot of each dilution was plated onto minimal glucose + ampicillin plates and were incubated at 37°C for two days. Two hundred colonies of each were gridded onto fresh minimal glucose + ampicillin plates. These were incubated at 37°C for two days. Plates were replica plated first onto minimal glucose + x-gal + p-gal + ampicillin

plates, then onto minimal glucose + ampicillin + chloramphenicol plates. The first set of plates was incubated at 37°C for one week; the second set, for two days. Colonies which were chloramphenicol sensitive were identified. The phenotype of these colonies on minimal glucose + x-gal + p-gal + ampicillin plates was observed. SPR112 which had had plasmid (pACYC184 + lambda #1) remained white whereas colonies which had had plasmid (pACYC184 + lambda #6) papilated. In other words, without plasmid (pACYC184 + lambda #6), SPR112 resumed its mutator phenotype. Because plasmid (pACYC184 + lambda #6) was derived from Kohara's lambda phage #347, the plasmid was designated as pKL347.

The plasmid pKL347 was reintroduced into SPR112 and the number of Lac^+ revertants was determined (for protocol, refer to ***PART 4: DOES DCM COMPLEMENT MUTG***).

Sequencing the lambda insert in pKL347

Essentially, the *E. coli* fragment which complements the mutation in SPR112 was cloned into M13 phage and was sequenced.

(a) Cloning the E. coli fragment from pACYC184 into M13

Various restriction enzyme digests of pKL347 revealed that the *Bam*HI site in which the *Sau*3A partials of KOHARA lambda clone 347 were introduced had not been restored. Consequently, plasmid pKL347 was digested with restriction enzymes *Hin*dIII and *Sal*I. *Hin*dIII and *Sal*I are located on

either side of the *Bam*HI cut site in pACYC184. Further, previous digestions of (pACYC184 + lambda #6), or pKL347, indicated that there were no *Hin*dIII and *Sal*I cut sites in the insert. The resulting fragment was cloned into phage M13mp18 and M13mp19 so as to be able to sequence both strands.

In microtube #1: 10 μl pKL347 plasmid
1 μl RNAse
1 μl *Hin*dIII 10 X Buffer
1 μl *Sal*I 10 X Buffer
10 U *Hin*dIII
16 U *Sal*I
5 μl H_2O

In microtube #M18:	In microtube #M19:
3 ul M13mp18 (100mg\ml)	3 μl M13mp19 (100mg\ml)
1 μl *Hin*dIII 10X buffer	1 μl *Hin*dIII 10X Buffer
1 μl *Sal*I 10X Buffer	1 μl *Sal*I 10X Buffer
10 U *Hin*dIII	10 U *Hin*dIII
16 U *Sal*I	16 U *Sal*I
13 μl H_2O	13 μl H_2O

Microtubes were incubated in a 37°C water bath for three hours. To make sure the DNA had not degraded and that the digestion was complete, once the time had elapsed, 2 μl of loading dye was added to a 10 μl aliquot of each digestion and the samples were electrophoresed on a 1% agarose gel at a 125 volts.

(b) Ligation of lambda #6 into M13mp18 and M13mp19

The desired ratio of lambda #6 to M13 was 1:1. Looking at the band intensities on the 1% agarose gel and taking into consideration the fact that M13 was at least twice as big as lambda #6, to get the same amount, M13 band should be twice as bright as that of lambda #6. Twice the volume of lambda #6 to M13 phage was used.

In microtube lambda 18:	In microtube lambda 19:
10 μl digested pKL347	10 μl digested pKL347
5 μl digested M13mp18	5 μl digested M13mp19
2 μl 10X ligase Buffer	2 μl 10X ligase Buffer
4 U ligase	4 U ligase
1 μl H_2O	1 μl H_2O

Samples were left at room temperature overnight.

(c) Phage transformation of JM105

Competent cells of JM105 were made (for protocol, refer to ***PART 4: DOES VSR COMPLEMENT THE MUTATION***, section ***Making SPR112 competent***). These were transformed with the pKL347/M13 ligation described above.

Each pKL347/M13 ligation mixture was divided into two microtubes. To each sample, 300 μl of competent JM105 cells was added. Microtubes were incubated on ice for 30 minutes and were then transferred to a 42°C water bath for half an hour. Samples were briefly put on ice to cool prior to being transferred to small glass culture tubes where 200 μl of log phase JM105 cells, 50 μl of 2% x-gal, 4 μl of 200 mg/ml

stock of IPTG, and 3 ml of melted top agar kept warm at 50°C were added. Tubes were vortexed briefly and immediately were poured onto LB plates. Plates were incubated overnight in a 37°C incubator.

(d) Isolation of double-stranded (M13mp18 + lambda #6) and (M13mp19 + lambda #6) DNA.

The following describes the protocol used for one potential clone. The same procedure was repeated for at least 10 potential clones in both orientations.

A culture of JM105 was made in LB media and was incubated at 37°C until log phase. In a test tube containing 3 ml of LB media, 100 μl of log phase JM105 culture and a white isolated plaque of phage transformed JM105 were added. The test tube was incubated in a 37°C shaker incubator for at least four and a half hours. A 1.5 ml aliquot was put in a microtube and was centrifuged at full speed for two minutes at room temperture. Being careful not to disturb the pellet, the supernatant was transferred to a fresh microtube to be used later to prepare single-stranded phage for sequencing. The protocol for the isolation of double-stranded phage is the same as ***Rapid plasmid preparation*** technique described earlier in this seccion.

To determine if the white plaques of M13 indeed had lambda #6 cloned into it, an aliquot of the double-stranded DNA preparation was digested with *Hin*dIII and *Sal*I and electrophoresed on a 1% agarose gel.

In microtube:

6 μl double stranded M13 + lambda #6 prep.

1 μl RNAse

1 μl *Hin*dIII 10X Buffer

1 μl *Sal*I 10X Buffer

10 U *Hin*dIII

16 U *Sal*I

Samples were incubated at 37°C for at least one hour. Two μl of loading dye was added to the sample which was electrophoresed on a 1% agarose gel at 125 volts. Single stranded phage preparations were made of the samples which had lambda #6 cloned into M13.

Preparation of single stranded phage DNA.

To the supernatant isolated during the ***Isolation of double-stranded (M13mp18 + lambda #6) and (M13mp19 + lambda #6) DNA*** described above, 200 μl of 20% polyethylene glycol (PEG)\ 2.5M sodium chloride was added. The sample was left at room temperature for 15 minutes. Then, the sample was centrifuged at full speed for 5 minutes at room temperature. The supernatant was aspirated. 100 μl of TE (10mM Tris·Cl\1mM EDTA) and 100 μl phenol were added to the microtube which was repeatedly vortexed until the pellet had completely dissolved. The sample was centrifuged at full speed for three minutes at room temperature. The top layer was transfered to a fresh microtube where 10 μl of 3M sodium acetate (pH 4.8) and 200 μl cold ethanol were added. Samples

were stored at -20°C overnight. The following morning, samples were centrifuged at full speed for thirty minutes at room temperature. The ethanol was aspirated. The pellet was dried in the lyophilizer and was resuspended in 25 μl of water. A 10 μl aliquot was used for sequencing. The primers used for the sequencing of the *E. coli* insert in pACYC184, now cloned into M13 phage, were designed as follows: nucleotide sequences on either side of the *Bam*HI restriction enzyme cut site in the plasmid pACYC184 (ROSE, 1988) that contained an equal number of purines and pyrimidines were chosen. These primers were designated primer **PACYC:1848** and **PACYC:1893**. Sequencing reactions were performed as described in the Pharmacia ^{T7} SEQUENCING ^{TM} KIT.

TABLE 1: LIST OF BACTERIAL STRAINS

Strain	Genotype
12068[1]	*zeb-3190/Tn10*
12099[1]	*zee-3129/Tn10*
12156[1]	*uvrC279/Tn10*
12179[1]	*mgl-500/Tn10*
18451[1]	*zed-3069/Tn10*
18467[1]	*zfb-1/Tn10*
CC112[2]	*ara* Δ*(lac pro) gyrA argE-am rpoB thi*; F^+:*proA,B*; *lacZ*$^-$, *lacY,A*; pGFIB(GluA)[3]
JM105[4]	*supE endA sbcB15 hsdR4 rpsL thi* Δ*(lac-proAB)*
JM801[5]	*ara* Δ*(lacproB) thi rpsL* (and F^+*kan*)
LE392[4]	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*
P90C[6]	*ara* Δ*(lac proB)*$_{XIII}$
RP4182[7]	*(flaD - flaP)* DE4 *trp gal rpsL* deleted for *dcm* and *supD*
S90C[5]	*ara* Δ*(lac pro) thi rpsL*
XAC[8]	*ara* Δ*(lac pro) gyrA argE-am rpoB thi*

[1] SINGER *et al.*,1989.

[2] CUPPLES, unpublished.

[3] KLIENA *et al.*, 1990.

[4] SAMBROOK *et al.*, 1982.

[5] CUPPLES and MILLER, 1988.

[6] CUPPLES and MILLER, 1989.

[7] SOHAIL *et al.*, 1990.

[8] COULONDRE and MILLER, 1977.

TABLE 2: STRUCTURE AND FUNCTION OF F' EPISOMES FROM CC101 TO CC106

Episome	*lacZ* sequence codons 460-462[1]	change at 461 for Lac$^+$	mutation detected
F' CC101[4]	AAT TAG ACT	TAG to GAG	T·A to G·C
F' CC102[4]	AAT GGG ACT	GGG to GAG	G·C to A·T
F' CC103[4]	AAT CAG ACT	CAG to GAG[2]	C·G to G·C
F' CC104[4]	AAT GCG ACT	GCG to GAG	C·G to A·T
F' CC105[4]	AAT GTG ACT	GTG to GAG	T·A to A·T
F' CC106[4]	AAT AAG ACT	AAG to GAG[3]	A·T to G·C

[1] Codons 460 to 462 in wild type *lacZ*: AAT GAG TCA

[2] also CAG to TAG in presence of glutamic acid inserting amber suppressor.

[3] also AAG to TAG in presence of glutamic acid inserting suppressor.

[4] Reference: CUPPLES and MILLER, 1989.

TABLE 3: LIST OF PRIMERS

Primer	Nucleotide Sequence
DCM:-007	5'-CGG CAT GCG ATC ATT TCC AGA CTA AGT TG-3'
DCM:1619	5'-CGG TGG CGT TGC GTC AGC AAG AGG CAC AA-3'
PACYC:1848	5'-TGG CGA CCA CAC CCG TCC T-3'
PACYC:1893	5'-ACG ATG CGT CCG GCG TAG A-3'
VSR:1806	5'-ACA AAA TCC GGA CGT CCG-3'
VSR:1951	5'-CAG TCG CTT GCA GGA ACT-3'
VSR:2145	5'-TAG TTG CGC CAG TTA TTC AGG ACG CAT CAA-3'
VSR:2262	5'-GCG GAT CCG GAC TGG GTG GAG AAA CAC-3'

A genetic selection system capable of isolating *E. coli* very short patch (VSP) repair mutators was designed and one such mutator, SPR112, was isolated in the lab by C. G. Cupples. To enable the reader to understand the logic behind the experiments done to characterize this mutant, the selection system and the procedure used to isolate SPR112 are described.

Genetic selection system used to isolate E. coli VSP repair mutators

This genetic selection system is based on the use of the *lacZ* gene to monitor VSP repair. In *E. coli*, the *lacZ* gene codes for the enzyme ß-galactosidase which catalyzes the cleavage of the disaccharide lactose into two monosaccharides, glucose and galactose, thus allowing the organism to use lactose as a carbon source. LANGRIDGE and CAMPBELL, 1969, showed that 93% of the point mutations in the *lacZ* gene which prevented growth on lactose plates were due to nonsense mutations rather than missense mutations. Likewise, close to two thirds of the selected nitrosoguanidine-induced mutants which had less than 50% of wild type ß-galactosidase activity were due to nonsense rather than missense mutations (LANGRIDGE, 1974). These studies suggest that most missense mutations have little effect on the activity of ß-galactosidase (CUPPLES and MILLER, 1988).

CUPPLES and MILLER (unpublished data cited in CUPPLES and MILLER, 1988; RUIZ, BIOL. 490 thesis, 1990) found that

random alterations in the base sequence of ß-galactosidase have little effect on enzyme activity. They suggested that the tolerance of the enzyme ß-galactosidase to changes in its primary sequence may be due to its large size of 1023 amino acids. Consequently, they proceeded to use site-directed mutagenesis to study the effect of amino acid substitutions at three positions believed to be part of the active site of ß-galactosidase. Their results support previous evidence that the glutamic acid residue at position 461 and the tyrosine residue at position 503 are essential for catalysis and suggest that the histidine residue at position 464 is not part of the active site but may be involved in substrate binding.

The designed assay system which is capable of isolating VSP mutators is summarized in ***FIGURE 5***. It is based on the fact that the glutamic acid residue at codon 461 of enzyme ß-galactosidase in *E. coli* is essential for a functional enzyme. Missense or nonsense mutations at 461 are Lac^-. In the wild type *lacZ* gene, codons 460 to 462 are: **AAT GAG TCA** (Asn Glu Ser). Site-directed mutagenesis was used to introduce the VSP target sequence at codons 460 to 462 which now reads **AAC CAG** GGG (Asn Gln Gly). The substitution of a glutamine for a glutamic acid at codon 461 renders the cell Lac^- or unable to use lactose as a carbon source. The product of the deoxycytosine methylase gene, *dcm*, methylates the second cytosine in the VSP target sequence, resulting in a 5-methylcytosine ($^{5\text{-me}}C$) (MARINUS, 1984). Whenever a $^{5\text{-me}}C$

deaminates, producing a thymine, a nonsense mutation (TAG) results. A nonsense mutation, or chain termination mutation, occurs when a point mutation results in one of the three termination codons in the middle of a gene. In this case, the C·G to T·A transition mutation results in an amber codon (TAG) at 461. An amber codon is one of three codons which does not represent amino acids. These are referred to as nonsense or termination codons and as their name implies, their function is to end protein synthesis. When an amber codon occurs within a gene, only a fragment of the wild-type protein is synthesized because there is no tRNA molecule whose anticodon is complementary to UAG. Fragments such as these have little if any biological function since only the first part of the protein is made in the mutant cell (LEWIN, 1987). Therefore, CAG to TAG should be Lac^- to Lac^- if it were not for the presence of a glutamic acid inserting suppressor. The glutamic acid suppressor inserts a glutamic acid in response to an amber codon because suppressor strains produce an altered tRNA capable of introducing an amino acid in response to a nonsense mutation. Cells are now Lac^+. Due to their inability to repair T·G mismatches, VSP mutators should have a high Lac^- to Lac^+ reversion frequency.

Isolation of a VSP repair mutant

The starting strain was CC112 (refer to ***FIGURE 6***). It has three different genetic elements: the chromosome, an

episome (F'CC112), and a plasmid (pGFIB). The chromosome has the entire *lac* operon deleted, is *pro*$^-$, and has an amber mutation in the *argE* gene. The F'CC112 episome has the *lac* operon and proline gene. The VSP target sequence, CCAGG, is centered around codon 461 of the *lacZ* gene. The plasmid pGFIB has the genes coding for the glutamic acid suppressor and ß-lactamase gene, *bla*, which provides the strain with an ampicillin resistant phenotype. To keep all the required components in the cell, the strain was kept on minimal glucose plates + ampicillin plates. The advantage in using minimal glucose as growth media was two-fold. First, the requirement for proline prevented the loss of the F'CC112 episome; second, although the ampicillin selected for the plasmid pGFIB, the arginine requirement for growth could only be met as long as the amber suppressor was still functional in pGFIB.

Random mutagenesis was used to inactivate a putative VSP repair gene. The chemical mutagen ethylmethane sulfonate (EMS) was used to induce random G·C to A·T transitions (MILLER, 1985). The logic behind using random mutagenesis relies on what LEATHERBARROW and FERSHT (1986) refer to as a *"one hit"* approach where it is very unlikely that more than one base change would be made in any given gene. Assuming that the mutagen caused a non lethal genetic change, the result of the mutation could be one of the following: (1) The mutation occurred in a gene that was not involved in VSP repair; (2) the mutagen caused a genetic change in a gene

that is involved in VSP repair, however, the mutation occured at a site which is very tolerant of base changes; consequently, the change does not hinder VSP repair; or (3) the mutagen caused a genetic change that could not be tolerated, such as a nonsense mutation, in a gene involved in VSP repair; consequently, VSP repair would no longer be functional. The assay system described above allows us to identify cells deficient in VSP repair because if VSP repair is functional in the mutagenized cell, the deaminated $^{5\text{-me}}C$ would be repaired and the original CCAGG sequence at codon 461 of the *lacZ* gene in the F'CC112 episome would be restored; the colony would have a Lac^- phenotype. However, if VSP repair would be inactivated due a mutation induced by the chemical mutagen EMS, the thymine which result from the deamination of $^{5\text{-me}}C$ would not be repaired. An amber codon would now be located at codon 461 of the *lacZ* gene and it would be suppressed by the glutamic acid suppressor in pGFIB. To summarize, VSP mutators would have a high frequency of Lac^+ reversion. To detect cells with a high rate of reversion cells were plated on papillation media which consists of: minimal glucose + 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (x-gal) + phenyl-ß-D-galactopyranoside (p-gal) + ampicillin. The chromogenic substrate (ie. a substrate which when cleaved yields a colored product) x-gal is colorless. However, when it is hydrolyzed by ß-galactosidase, a blue compound (5-bromo-4-chloro-indigo) is released. Cells grow using glucose as a carbon source until the glu-

cose is exhausted. Lac^+ cells in a colony then continue to grow using x-gal and p-gal as carbon sources; further, x-gal stains these Lac^+ cells blue. Colonies derived from cells with a high Lac^+ reversion frequency due to a defective VSP repair system should have a high number of papillae. One candidate was isolated, SPR112.

To quantify the qualitative papillation assay, overnights of SPR112 were plated on minimal lactose + ampicillin plates. ***TABLE 4*** and ***FIGURE 7*** compare the Lac^+ reversion rate of SPR112 with CC112, the wild type strain from which SPR112 was derived. The number of Lac^+ colonies per 10^8 viable cells when CC112 and SPR112 were plated on minimal lactose + ampicillin plates was 0.2 and 67 respectively. SPR112 has approximately a 345 fold higher frequency of Lac^+ revertants than its wild type counterpart.

PART 1: THE SPECIFICITY OF SPR112

A mutator strain, SPR112, with a high Lac^+ reversion frequency was isolated by C.G. CUPPLES. The purpose of this project was to characterize this mutant. It appeared to have a high rate of C·G to T·A transition mutations at the second C in the base context CCAGG. However, Lac^+ phenotype could also result from C·G to G·C tranversions (CAG to GAG). To determine which base change was indeed happening in SPR112, the F'CC112 episome was isolated from Lac^+ revertants and was transferred to a nonsuppressor strain, S90C. If the

cells were indeed CAG to TAG revertants, without the glutamic acid inserting suppressor, the colonies would remain white when plated on media supplemented with x-gal. However, if the revertants were spontaneous CAG to GAG mutants, colonies would appear blue on media containing x-gal. The recipients remained white (data not shown), indicating that the episomes contain a TAG codon not a GAG codon at 461. In other words, SPR112 stimulates C·G to T·A transition mutations not C·G to G·C transversions.

If SPR112 is a new mutator, can it cause any other kinds of mutations other than C·G to T·A transitions at the internal C of the nucleotide sequence CCAGG? Conversely, if it is not a new mutator, is it a strain with a defect in a gene whose product is required for the correction process of a system other than VSP repair? For example, inactivation of any of the genes required for the *dam*-directed mismatch repair system could also account for this high frequency of C·G to T·A transitions. However, a deficiency in the methyl-directed mismatch repair system would stimulate C·G to T·A transition mutations regardless of sequence context along with a number of other transition and transversion mutations whereas a strain defective in VSP repair could only exhibit a high frequency of C·G to T·A mutations at the second C of the nucleotide sequence CCAGG.

To determine the specificity of the mutations generated by SPR112, SPR112 was cured of its F'CC112 episome and was replaced with one of six tester episomes, F'CC101 to F'CC106

(MILLER, 1972). The new strains were designated SPR101 to SPR106, respectively. Each episome monitors a different mutation; for the list of mutations, refer to ***TABLE 2: STRUCTURE AND FUNCTION OF F' EPISOME FROM CC101 TO CC106.*** To determine the number of Lac^+ revertants per 10^8 viable cells, overnight cultures of SPR101 to SPR106 were made and these were plated on lactose minimal media + ampicillin and cn LB plates (CUPPLES and MILLER, 1989). Results of the experiment are recorded in ***TABLE 4: LAC^+ REVERSION FREQUENCIES FOR STRAIN CC112 AND SPR112 WITH THE SEVEN F'CC EPISOMES***, illustrated in ***FIGURE 7***, and are described below.

To establish if the high levels of G·C to A·T mutations in SPR112 were restricted to the internal C of the VSP target sequence CCAGG, the F'CC102 episome was introduced into SPR112 (Note: SPR112 had been cured of episome F'CC112). F'CC102, like F'CC112, monitors C·G to T·A transitions but in a non-VSP sequence context. For CC102 and SPR102, the # Lac^+ revertants/10^8 cells was 0.32 and 1.23 respectively. Although the frequency of Lac^+ revertants is higher for SPR102 than CC102, is it two orders of magnitude lower than that for SPR112. If SPR112 is defective in VSP repair, the elevated levels of C·G to T·A transversions in the cell may be too high for the methyl-directed system to correct. Thus, the observed increase in SPR102, compared to its wild type counterpart (CC102) may be the result of an overload of the *dam* correction system. These results demonstrate that SPR112 stimulates C·G to T·A transitions at the

second C of the nucleotide sequence CCAGG.

To monitor the reverse transversion mutation (T·A to G·C), F'CC112 was replaced with the F'CC101 episome. The F'CC101 episome has an amber (TAG) codon at 461. In the presence of the suppressor, all cells are Lac^+. To monitor A·T to C·G transversion, SPR101 was cured of pGFIB, the plasmid which carries the glutamic acid inserting suppressor, and the number of Lac^+ revertants per 10^8 cells was determined. The Lac^+ reversion frequency of SPR101(Su^-) is as low as CC112, the wild type strain from which SPR112 was derived. The results indicate that SPR112 does not stimulate A·T to C·G mutations.

In SPR103, the F'CC103 episome monitors C·G to G·C mutations. The Lac^+ reversion frequency of SPR103, 5.40, is approximately 27 fold higher than that of CC103 or CC112. This relatively elevated reversion frequency could be attributed to the fact that F'CC103 monitors not only C·G to G·C mutations but also C·G to T·A transitions, giving rise to an amber codon at 461 which in the presence of the glutamic acid inserting suppressor, becomes Lac^+. In other words, the frequency of Lac^+ revertants for SPR103 is most likely a combination of the two types of mutations described. One way to distinguish between the two mutations would be to use the same approach described in SPR101, to remove the suppressor plasmid, pGFIB from the strain so as to construct SPR103(Su^-). The reversion frequency was low enough that such a strain construct was not made.

Episome F'CC104 monitors C·G to A·T mutations. The Lac^+ reversion frequency of SPR104 is slightly higher than wild type levels; however, this slightly elevated level is consistent with the F'CC104. Other investigators have found that whenever F'CC104 has been introduced into a strain that was subject to mutagens, oxidative or metabolic stress, the mutational level was elevated (CUPPLES, personal communications). This elevation was not seen when one of the other F'CC episomes was used.

Based on the wild type levels of Lac^+ reversion frequencies of SPR105 and SPR106, SPR112 does not stimulate T·A to A·T or T·A to C·G mutations.

To summarize, results of this experiment indicate that SPR112 stimulates primarily C·G to T·A transitions at the second C of the sequence CCAGG.

PART 2: MAPPING THE MUTATION.

After SPR112 was isolated by C.G. CUPPLES and its mutator specificity was determined by myself, one gene shown to be involved in VSP repair, *vsr*, was identified in another laboratory. *Vsr* maps at 43 minutes on the *E. coli* chromosome (SOHAIL *et al.*, 1990). The first attempt to determine if the mutator phenotype of SPR112 was due to a mutation in *vsr* was to determine if the mutation mapped at 43 minutes. The mutation was mapped by P1 transduction. The strains of the SINGER mapping kit (SINGER *et al.*, 1989) which span 41.25

through 45.75 minutes on the *E. coli* chromosome, inclusive, were used; strain CAG18467, which maps at 51.00 minutes was used as a control. Phenotypically, a wild type strain may have 1 papillae, two at the most, per colony; a colony contains about 10^8 cells. This is consistent with the estimated replication fidelity of one misincorporated base per 10^9 to 10^{10} base replicated (DRAKE, 1969 cited in SCHAAPER and RADMAN, 1989). Based on this, a colony with more than three papillae was classified as a papillator. The total number of transductants was recorded. The percentage of nonpapillators to the total number of colonies was calculated. Results are recorded in ***TABLE 5: GENETIC MAPPING OF MUTG: P1 TRANSDUCTION RESULTS***. 88.7% of the colonies transduced with CAG18451 were not papillators, compared with 24.5% with the control strain. However, for the other strains used, the percentage of nonpapillators hovered near 50%. The colonies were restreaked onto fresh plates. Interestingly enough, some of these "nonpapillators" began to papillate! These were counted and the new percentage of nonpapillators to the original number of total colonies was calculated. The number of nonpapillators for the control strain decreased to 1.7% from the initial 24.5%. Over a 5 fold decrease in the number of nonpapillators was recorded for the other mapping strains except strain CAG18451. 81.0% of the transductants were nonpapillators. When streaked onto fresh plates, nonpapillators remained nonpapillators. The results of this experiment suggest that the mutation, like *vsr*, maps at 43 minutes.

Further, the mapping results prove that the mutation is not in the methyl-directed mismatch repair system; genes required for the *dam*-directed repair process -*dam* (74 min.), *mutH* (61 min.), *mutL* (95 min.), *mutS* (59 min.), and *mutU* or *uvrD* (86 min.) - do not map at 43 minutes on the *E. coli* chromosome (BACHMANN, 1990). The interval between CAG18451 (43.00 min.) and CAG12099 (44.25 min.) is about a 100 kb interval (approximately 80 kb/min.). In *E. coli*, the average size of a gene is about 1 kb; this region is therefore likely to contain a large number of genes. Moreover, it is not uncommon to find genes that are involved in the same system to map close to one another. For example, in *E. coli* a cluster of three genes constitutes the *lac* operon. Although the protein product of the *lacA* gene (thiogalactoside transacetylase) is not essential for cell growth or for the utilization of lactose as a carbon source, the *lacZ* and the *lacY* gene products (ß-galactosidase and lac permease, respectively) are necessary for lactose metabolism.

PART 3: IS THE DEOXYCYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112

The activity of cytosine methylation in SPR112 was verified to make sure that SPR112 was not *vsr*$^-$ due to a mutation in the regulatory region which also controls *dcm*.

The purpose of this experiment was to determine if SPR112 had a functional deoxycytosine methylase gene, *dcm*.

The following strains were used: CC112, RP4182, and SPR112. CC112 and RP4182 were used as controls. CC112 has a functional *dcm* gene whereas RP4182 is Dcm$^-$ due to a chromosomal deletion at 43 minutes which includes *dcm*. Chromosomal DNA was isolated from the three strains and was digested with the restriction enzyme *Eco*RII. *Eco*RII cuts unmethylated CCAGG sequences only; methylated CCAGG sequences cannot be digested with *Eco*RII. A 1% agarose gel of the digested samples revealed that only chromosomal DNA from RP4182 was cut by *Eco*RII whereas chromosomal DNA isolated from CC112 and SPR112 was not digested by *Eco*RII (refer to ***FIGURE 8: IS THE DEOXYCYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112?***). These results suggest that the deoxycytosine methylase gene in SPR112 is fully functional.

PART 4: DOES VSR COMPLEMENT THE MUTATION?

To determine if the mutation was in *vsr*, a complementation analysis was done. Plasmid pDCM28, courtesy of A.S. Bhagwat, contains only the promoter region of *dcm* and the *vsr* gene cloned into the *Sph*I-*Bam*HI site of pACYC184; the *dcm* gene had been inactivated by deleting the *Ava*I-*Cla*I fragment. It has been previously shown that pDCM28 restores VSP repair, but not cytosine methylation, in a *dcm*$^-$*vsr*$^-$ (*dcm-6*) strain (SOHAIL *et al.*, 1990). Competent cells of SPR112 were transformed with plasmid pDCM28. Results are recorded in ***TABLE 6: COMPLEMENTATION ANALYSIS***. Approximately

a five fold decrease in the number of Lac^+ revertants was observed when SPR112 was transformed with pDCM28. However, a five fold decrease was also observed by simply transforming SPR112 with the plasmid pACYC184, the plasmid from which pDCM28 was derived. These results suggest that the decrease in Lac^+ revertants is due to the presence of the plasmid in SPR112 and not due to complementation. These results suggest that the mutation is not in *vsr*.

PART 5: SPR112'S VSR GENE, WILD TYPE OR MUTANT?

To determine if the *vsr* gene from SPR112 and CC112 was wild type, the gene's nucleotide sequence was determined.

The wild type nucleotide sequence of the *dcm-vsr* genes had previously been published (SOHAIL *et al.*, 1990); based on this sequence, primers **VSR:2262** and **VSR:-007** were designed (for the nucleotide sequence of these primers, refer to ***TABLE 3: LIST OF PRIMERS***). These primers were used to amplify the *dcm-vsr* genes, from SPR112 and CC112, by the polymerase chain reaction (PCR) technique. The *vsr* gene was sequenced from the amplified product directly. This was done because the *Taq* DNA polymerase lacks 3' to 5' editing function; misincorporation of bases occurs at an estimated frequency of 2×10^{-4} nucleotides per PCR cycle (SAMBROOK *et al.*, 1982). These misincorporations occur throughout the length of the amplified product. Therefore, sequencing an individual DNA molecule cloned from the amplified pool is

not reliable. One alternative would be to have sequenced a number of clones generated from separate amplification reactions. However, the option of sequencing from a pool of single-stranded DNA was chosen. Each strand of the *dcm-vsr* gene was assymmetrically amplified, and using primers **DCM:1619** and **V8R:2145** (the nucleotide sequence of these primers is recorded in ***TABLE 3: LIST OF PRIMERS***), the *vsr* gene was sequenced. The nucleotide sequence obtained was compared to the wild type nucleotide sequence of *vsr*. To date, 99.6% of *vsr* in SPR112 has been sequenced; this portion of the gene is wild type.

PART 6: THE FINER MAPPING AND CLONING OF MUTG

KOHARA (KOHARA *et al.*, 1987) published a restriction enzyme map that covers the whole *E. coli* genome and it also is related to the clone bank he constructed. Lambda phage clones which spanned the 43 minute region were used. ie: #343 to 351. These were partially digested with *Sau*3A; fragments were cloned into the *Bam*HI site of plasmid pACYC184 and then transformed into competent cells of SPR112. Transformants were plated onto minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates. After a one week incubation period, only the 544 colonies which had less than three papillae were subject to further analysis.

To determine if the subclones introduced into SPR112 which exhibited less than three papillae indeed had a lambda

DNA fragment cloned into the *Bam*HI site of its tetracycline gene, the colonies were gridded onto fresh minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates. Plates were then replica plated first onto a fresh plate of the same media and then onto a minimal glucose + ampicillin + tetracycline plate. Only 197 of the 544 colonies were tetracycline sensitive; these were selected. After the one week incubation on the minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates, only six of the 197 colonies retained their "less than three papillae" phenotype; these were designated as SPR112 + (pACYC184 + lambda) #1 to #6. However, when these strains were restreaked onto fresh minimal glucose + x-gal + p-gal + ampicillin + chloramphenicol plates, SPR112 + (pACYC184 + lambda) #4 and #5 papillated. These were no longer used.

To further determine if (pACYC184 + lambda #1, #2, #3, and #6) indeed did have a fragment cloned into its *Bam*HI site, the transformants were first cured of the second plasmid, pGFIB. An overnight culture of each SPR112 + (pACYC184 + lambda) #1, #2, #3, and #6 in LB + chloramphenicol; no ampicillin was used so as to not select for the plasmid pGFIB. Further, the use of rich media did not select for the glutamic acid inserting suppressor. Plasmid preparations were made of each culture. Competent cells S90C were transformed with each individual pGFIB/(pACYC184 + lambda) mixture. Transformants were plated onto LB + chloramphenicol plates; again, growth conditions favored the loss of plasmid

pGFIB. From each plate, 50 transformants were randomly chosen and were gridded onto fresh plates. Plates were replica plated onto LB + ampicillin plates. Colonies which were chloramphenicol resistant and ampicillin sensitive were identified and purified for single colonies. These colonies contained only (pACYC184 + lambda) plasmid and not pGFIB. The plasmid was isolated from each of these colonies. It was digested with various restriction enzymes and was electrophoresed on a 1% agarose gel.

To determine if the plasmids had an insert, they were digested with *Hin*dIII and *Sph*I and the samples were electrophoresed on a 1% agarose gel. Of the four plasmid preparations, only (pACYC184 + lambda) #1 and #6 had inserts. Fortunately, these inserts did not have *Hin*dIII or *Sph*I cut sites; the size of the insert was approximately 2 kb.

To establish if the decrease in Lac^+ revertants in SPR112 + (pACYC184 + lambda) #1 and #6 was due to complementation of the mutation by the clone derived from the lambda fragment, the strains were cured of the pACYC184 derived plasmid.

Overnight cultures of SPR112 + (pACYC184 + lambda) #1 and #6 were made in minimal glucose + ampicillin solution; to favor the loss of (pACYC184 + lambda) plasmid, no chloramphenicol was included. The overnight culture was diluted to 10^{-6} and a 100 μl aliquot of each dilution was plated onto minimal glucose + ampicillin plates. Two hundred colonies of each were gridded onto fresh plates. Once the colonies had

grown, plates were replica plated first onto minimal glucose + x-gal + p-gal + ampicillin plates, then onto minimal glucose + ampicillin + chloramphenicol plates. The first set of plates were incubated at 37°C for one week; the second set, for two days. Colonies which were chloramphenicol sensitive were identified. The phenotype of these colonies on minimal glucose + x-gal + p-gal + ampicillin plates was observed. SPR112 which was cured of plasmid (pACYC184 + lambda #1) remained white whereas colonies which were cured of plasmid (pACYC184 + lambda #6) papillated (data not shown). These results suggest that the low Lac^+ reversion frequency of SPR112 + (pACYC184 + lambda #1) is not due to the presence of the insert in pACYC184. However, without plasmid (pACYC184 + lambda #6), SPR112 resumed its mutator phenotype indicating that the mutation is complemented by the fragment cloned into pACYC184. Because plasmid (pACYC184 + lambda #6) was derived from Kohara's lambda phage #347, the plasmid was designated as pKL347.

The plasmid pKL347 was reintroduced into SPR112 and the number of Lac^+ revertants was determined (for data, refer to ***TABLE 6:COMPLEMENTATION ANALYSIS OF MUTG***). The presence of plasmid pKL347 in SPR112 decreased the frequency of Lac^+ revertants to wild type levels. Results of this experiment suggest that the mutator gene in SPR112 has been cloned into pACYC184.

SOHAIL and coworkers (1990) aligned the restriction enzyme pattern predicted from their nucleotide sequence of

dcm-vsr with KOHARA's 4700 kb integrated restriction map. Based on the map position of *dcm* and *vsr*, they suggest that the genes lie in phage lambda clone 344. The *E. coli* fragment which complements the mutation in SPR11? was derived from the lambda phage clone 347; approximately 25 kb separates these two fragments. These results along with the complementation analysis and the sequence of *vsr* in SPR112 suggest that the mutation in SPR112 is not in *vsr* but in another gene which is also required for VSP repair; this gene will be referred to as *mutG*.

Sequencing the cloned fragment is currently under way. Various restriction enzyme digests of pKL347 revealed that the *Bam*HI sites were not restored when *Sau*3A partials were cloned into the *Bam*HI site of pACYC184. Consequently, pKL347 was digested with *Hin*dIII and *Sal*I. Previous experiments determined the insert had no cut sites for these enzymes and in pACYC184, these sites are on either side of the *Bam*HI site. The fragment was cloned into M13 in both orientations. Essentially, nucleotide sequences on either side of the *Bam*HI restriction enzyme cut site in the plasmid pACYC184 (ROSE, 1988) that contained an equal number of purines and pyrimidines were used as primers. These primers were designated primer **PACYC:1848** and **PACYC:1893**. For the nucleotide sequence of these primers, refer to ***TABLE 3: LIST OF PRIMERS***.

To date, 303 base pairs have been sequenced from one extremity of the insert. Comparison of the sequence to three

separate data bases revealed that this sequence does not contain lambda DNA or a fragment of plasmid pACYC184; further, this piece of *E. coli* DNA has not been associated with any other gene that maps at 43 minutes on the *E. coli* chromosome. Currently, sequencing the other extremity of the insert is underway.

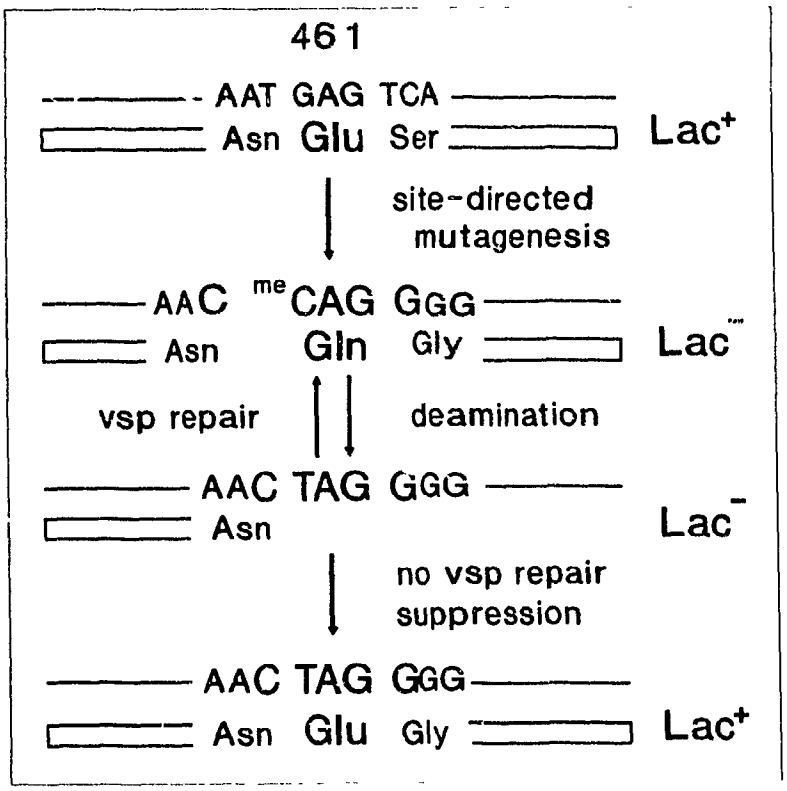

FIGURE 5: USE OF THE LACZ GENE TO MONITOR VSP REPAIR IN E. COLI. This assay system is based on the necessity of the glutamic acid residue at codon 461 of enzyme ß-galactosidase. Missense or nonsense mutations at 461 are Lac$^-$. In the wild type *lacZ* gene, codons 460 to 462 are: AAT **GAG** TCA (Asn Glu Ser). Site-directed mutagenesis was used to introduce the VSP target sequence at codons 460 to 462 which now reads AAC **CAG** GGG (Asn Gln Gly). The substitution of a glutamine for a glutamic acid at codon 461 renders the cell Lac$^-$. The product of gene *dcm* methylates the second cytosine in the VSP target sequence, resulting in a 5-methylcytosine. Whenever a 5-methylcytosine deaminates, producing a thymine, the nonsense codon, TAG, results. When an amber codon occurs within a gene, only a fragment of the wild-type protein is synthesized. Fragments such as these have little if any biological function (LEWIN, 1987). Therefore, CAG to TAG should be Lac$^-$ to Lac$^-$ if it were not for the presence of a glutamic acid inserting suppressor. The glutamic acid suppressor inserts a glutamic acid in response to an amber codon. Cells are now Lac$^+$. Due to their inability to repair thymine (or deaminated 5-methylcytosines), VSP mutators should have a high Lac$^-$ to Lac$^+$ reversion frequency.

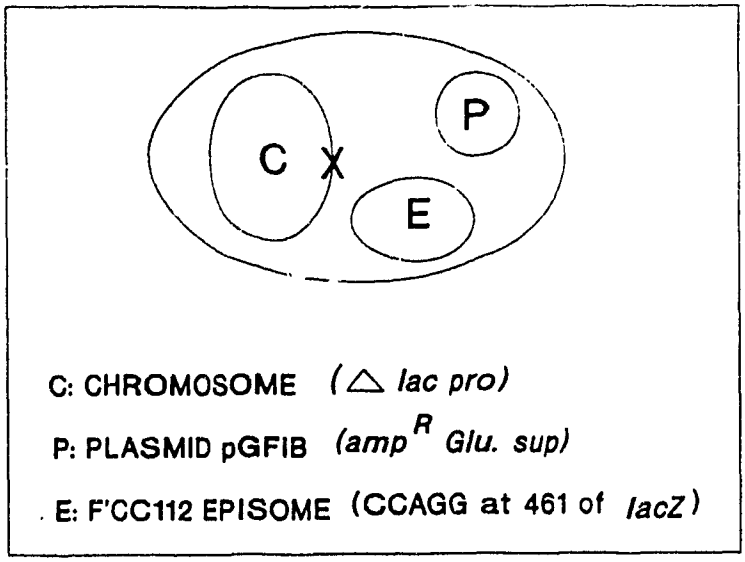

FIGURE 6: CC112: GENETIC ELEMENTS. CC112 has three different genetic elements: the chromosome, an episome (F'CC112), and a plasmid (pGFIB). The chromcsome has the entire *lac* operon deleted, is *pro*$^-$, and has an amber mutation in the *argE* gene. The F'CC112 episome has the *lac* operon and proline gene. The VSP target sequence, CCAGG, is centered around codon 461 of the *lacZ* gene. The plasmid pGFIB has the genes coding for the glutamic acid suppressor and ß-lactamase gene, *bla*, which provides the strain with an ampicillin resistant phenotype.

TABLE 4: LAC$^+$ REVERSION FREQUENCIES FOR STRAIN CC112 AND SPR112 WITH THE SEVEN F'CC EPISOMES

	CC112[1]	SPR112[1]
Episome	**# Lac$^+$/10^8 cells**	**#Lac$^+$/10^8 cells**
F'CC101	8.1 X 10^7 (n=4)[2]	1.0 X 10^8 (n=5)[2]
F'CC101(Su$^-$)	0.41 (n=3)[2]	0.21 (n=3)[2]
F'CC102	0.32 (n=6)[2]	1.23 (n=3)[2]
F'CC103	0.18 (n=5)[2]	5.40 (n=3)[2]
F'CC104	0.07 (n=6)[2]	1.27 (n=3)[2]
F'CC105	0.15 (n=6)[2]	0.27 (n=3)[2]
F'CC106	0.14 (n=5)[2]	0.30 (n=5)[2]
F'CC112	0.2 (n=5)[2]	67.0 (n=4)[2]

[1] F'CC112 episome was removed prior to the independent introduction of F'CC101 to F'CC106.

[2] Unless the viability was at least 10^9 cells per ml, the data was discarded.

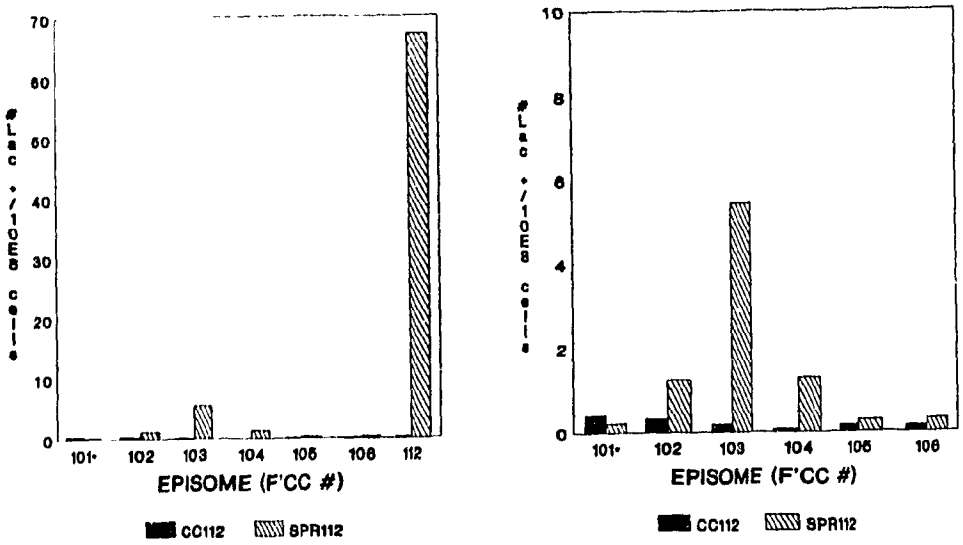

FIGURE 7: BAR GRAPH: LAC$^+$ REVERSION FREQUENCY FOR STRAIN CC112 AND SPR112 WITH THE SEVEN F'CC EPISOMES. CC112 was cured of its F'CC112 episome and it was individually replaced with all six tester episomes (F'CC101 to F'CC106), separately. The procedure was repeated with SPR112 and is as described in ***MATERIALS AND METHODS.*** * Cells transformed with F'CC101 were also cured of plasmid pGFIB. **LEFT:** Lac$^+$ reversion frequency of CC112 and SPR112 is compared along with CC101 to CC106 and SPR101 to SPR106. **RIGHT:** to enhance the effect of the six tester episomes in the different strain backgrounds, the Lac$^+$ reversion rate of CC112 and SPR112 was not included and the scale on the y-axis was reduced sevenfold.

TABLE 5: GENETIC MAPPING OF MUTG: P1 TRANSDUCTION RESULTS

DONOR STRAIN	Tn10 INSERT LOC.	TOTAL # COLONIES	% < 3 PAP.	RESTREAK % < 3 PAP.
CAG12068	41.25 min.	532	43.6	5.6
CAG12156	42.25 min.	347	50.4	11.0
CAG18451	43.00 min.	547	88.7	81.0
CAG12099	44.25 min.	256	48.4	6.6
CAG12179	45.75 min.	360	42.5	11.1
CAG18467	51.00 min.	290	24.5	1.7

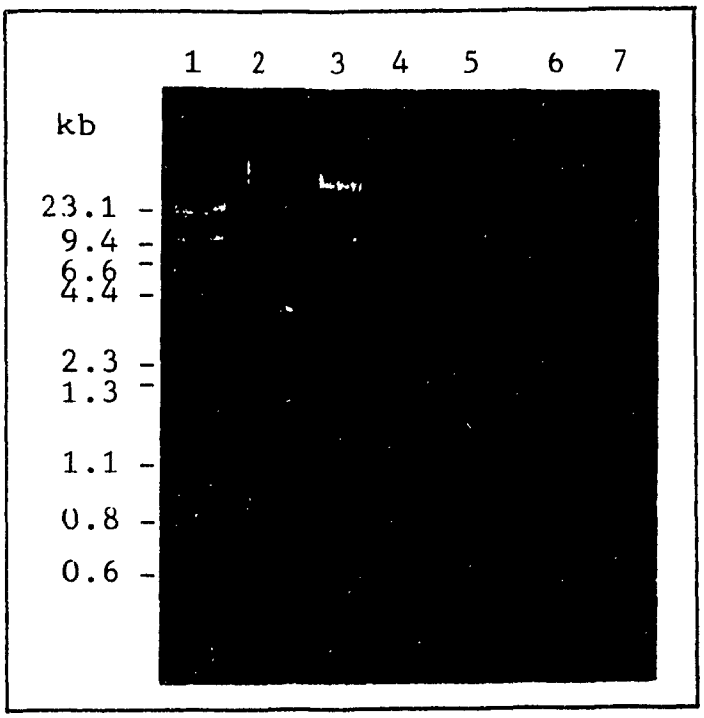

FIGURE 8: IS THE DEOXYCYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112?.
Lane 1: 1 μg of lamda DNA-*Hin*dIII/ΦX-174 DNA-*Hae*III digest marker. **Lane 2:** 1330 ng of CC112 chromosomal DNA. **Lane 3:** 1330 ng of CC112 chromosomal DNA treated with 2 U of *Eco*RII. **Lane 4:** 570 ng of RP4182 chromosoaml DNA. **Lane 5:** 570 ng of RP4182 chromosoaml DNA treated with 2 U of *Eco*RII. **Lane 6:** 725 ng of SPR112 chromosomal DNA. **Lane 7:** 725 ng of SPR112 chromosomal DNA treated with 2 U of *Eco*RII.

TABLE 6: COMPLEMENTATION ANALYSIS OF MUTG

Strain	Plasmid	#Lac$^+$/10^{8}cells
CC112	none	0.2[1]
SPR112	none	67
SPR112	pDCM28	12
SPR112	pACYC184	11
SPR112	pKL347	0.2[1]

[1] The reversion frequency for CC112 and for SPR112 + pKL347 was so low that 1 ml of overnight culture, as opposed to 100 μl which was used for the other strains, was plated onto minimal lactose media. This was done by centrifuging a 1 ml aliquot of the overnight culture for two minutes at room temperature, discarding the supernatant, resuspending the pellet in 100 μl of minimal lactose media, and plating the whole suspension onto a minimal lactose + ampicillin + chloramphenicol plate. The extra significant digit is due to the plating of this larger volume.

DISCUSSION

In *Escherichia coli*, the product of gene *dcm* methylates the second cytosine in the nucleotide sequence CC(A/T)GG. In DNA, deamination of 5-methylcytosines results in T·G mismatches. If unrepaired, these mismatches can lead to C to T transition mutations. The specific action of VSP repair to correct T·G mismatches in favor of the G-containing strand at cytosine methylation sequences suggests that VSP repair has evolved to counteract the effects of this mutagenic process. Essentially, the following approach was used: (1) a genetic selection system capable of monitoring VSP repair or the lack of VSP repair was designed; (2) using a chemical mutagen, a putative VSP repair gene was inactivated; (3) a strain, SPR112, exhibiting the characteristics of a VSP mutator was isolated; and (4) the mutator was characterized as described in the following paragraphs.

A potential *E. coli* VSP mutator, SPR112, was isolated. It has high levels of C·G to T·A transitions at CCAGG sequences. Elevated levels of C·G to T·A transitions could also be due to the inactivation of a gene required for the only other characterized *E. coli* repair system that can correct G·T base pairs: the methyl-directed repair system. Consequently, the specificity of SPR112 was determined.

PART 1: THE SPECIFICITY OF SPR112

Individual replacement of the F'CC112 episome with the six tester episomes, F'CC101 to F'CC106, demonstrated that

SPR112 exhibits the characteristics of a true VSP mutator: it induces only C·G to T·A transition mutations at cytosine methylation specific sequences. If SPR112 had been deficient in the methyl-directed repair system, elevated levels of C·G to T·A mutations would have been detected regardless of sequence context along with various other mutations (especially A·T to G·C) (SCHAAPER and DUNN, 1987).

After SPR112 was isolated, one gene shown to be required for VSP repair, *vsr*, was identified in another laboratory; *vsr* maps at 43 minutes on the *E. coli* chromosome (SOHAIL *et al.*, 1990). To determine if the mutator phenotype of SPR112 was the result of a mutation in *vsr*, the inactivated gene was mapped.

PART 2: THE MAPPING OF MUTG

Results of the P1 transduction experiment revealed that the mutated gene in SPR112, like *vsr*, maps at 43 minutes on the *E. coli* chromosome. These results also support one of the conclusions of the previous experiment: SPR112 is not defective in a gene involved in the *dam*-directed mismatch repair system because the defective gene does not map near any gene required for methyl-directed repair – *dam*, 74 min.; *mutH*, 61 min.; *mutL*, 95 min.; *mutS*, 59 min.; and *mutU* or *uvrD*, 86 min. The fact that the mutation in SPR112 maps at the same "minute" as *vsr* does not rule out the possibility that they are separate genes since there is a 100 kb inter-

val between mapping strains CAG18451 (43.00 min.) and CAG12099 (44.25 min.). It is also possible that their products may interact in the VSP repair process.

The 43 minute region of the *E. coli* chromosome was scanned for any identified gene that may have an unsuspected role in VSP repair. The *sbcB* or *xonA* gene, which maps close to the 44 minute interval, codes for an exonuclease I (BACHMANN, 1990). If a mismatch specific endonuclease cuts either 5' or 3' to the T·G mismatch, could this exonuclease, product of the *sbcB* gene, play a significant role in VSP repair by excising only a single or a few nucleotides? Only future experiments, such as comparison of the nucleotide sequence of the cloned fragment with that of the *sbcB* gene, could answer this question.

The activity of *dcm* in SPR112 was verified to make sure the mutator strain was not VSP$^-$ due to (1) a mutation in the regulatory portion of *dcm-vsr*, or (2) a polar mutation in *dcm*.

PART 3: IS THE DEOXYCYTOSINE METHYLASE GENE FUNCTIONAL IN SPR112?

The inability of *Eco*RII to digest chromosomal DNA isolated from SPR112 illustrated the functional activity of gene *dcm*; further, it also eliminated the possibility that SPR112 was *vsr*$^-$ due to a mutation in the regulatory portion of the gene because they are driven from the same promoter.

As previously discussed, methylation of cytosines by the product of gene *dcm* is potentially mutagenic. The specific action of VSP repair on cytosine methylation sequences suggests that VSP repair has evolved to minimize the mutagenic consequence of cytosine methylation. So the question: "why does *E. coli* methylate cytosines in the first place?" In mammalian cells, methylation of cytosines within CpG dinucleotides is believed to play an important role in the regulation of gene expression; no role has been attributed to cytosine methylation in *E. coli*. Knowing the function of methylated cytosines may help in understanding the cycle of methylation, deamination, and mismatch repair.

To determine whether SPR112 was VSP$^-$ due to a mutation within the *vsr* gene, a complementation analysis was done.

PART 4: DOES VSR COMPLEMENT THE MUTATION?

Plasmid pDCM28 consists of the wild type *vsr* gene, along with the promoter region of *dcm*, cloned into pACYC184. When pDCM28 was introduced into SPR112, the same decrease in the rate of Lac$^+$ revertants was observed as in SPR112 cells transformed with the control plasmid pACYC184 and was still greatly elevated compared to the wild type. The results suggest that the decrease in Lac$^+$ revertants is due to the presence of the plasmid and not due to complementation. The data indicates that the mutator phenotype of SPR112 is not due to a mutation in *vsr*.

One method that can help to determine whether the *vsr* gene in SPR112 is wild type is to sequence the gene from the mutator strain; alternative techniques include: (1) demonstrating the *vsr* gene from SPR112 is functional in a VSP^- strain (experiment in progress); or (2) isolate the *vsr* protein product from SPR112 and show that it is functional *in vitro* by doing heteroduplex experiments.

PART 5: SPR112'S VSR GENE, WILD TYPE OR MUTANT?

Using the polymerase chain reaction, the *dcm-vsr* genes were asymmetrically amplified from SPR112 and CC112, its wild type progenitor. The *vsr* genes from both strains were sequenced. The nucleotide sequence obtained was compared to the wild type sequence of *vsr*. To date, the sequenced 99.6% of the *vsr* gene in SPR112 is wild type.

If the complete sequence of the *vsr* gene in SPR112 proves to be wild type, the isolation of a novel mutator locus necessary for VSP repair would be implied.

PART 6: THE FINER MAPPING AND CLONING OF MUTG

Fragments of the 43 minute portion of the *E. coli* chromosome, derived from the KOHARA clone bank, were subcloned into pACYC184. These were individually introduced into SPR112. One transformant which exhibited a wild type phenotype along with wild type levels of Lac^+ revertants was

isolated. A fragment which complements the mutation in SPR112 had been cloned into pACYC184; this new plasmid was designated pKL347. This fragment was derived from a KOHARA phage different from the one which bears the *dcm-vsr* genes. A partial sequence of the cloned fragment showed no sequence similarity with any previously isolated gene.

To characterize *mutG*, the following remains to be done: (1) sequence the *E. coli* fragment which has been cloned into the *Bam*HI site of pACYC184; (2) search the sequence for open reading frames and potential regulatory regions; (3) clone the putative gene into pACYC184, omitting extra base sequences; (4) reintroduce plasmid into SPR112 and monitor Lac^+ reversion frequency. If it has decreased to wild type levels, (5) determine the role of *mutG* in VSP repair. One useful approach would be to compare the amino acid sequence to that of characterized genes to see if any similarities exist.

Caution must be taken when cloned genes are isolated based on their ability to complement the deficiency of a DNA repair mutant. As opposed to having isolated the wild type gene which corresponds to the one disrupted in the mutant, the cloned fragment may in fact be suppressing the mutator locus, for example, by increasing the efficiency of an alternative pathway or for some other reason (WALKER *et al.*, 1985). Again, determining how this gene interacts with the VSP correction system would provide insight as to the regulation of this correction process.

Once the cloned fragment in pACYC184 has been sequenced, one method that can be used to determine if this gene suppresses the mutation in SPR112 is the following: (1) disrupt the gene cloned in the plasmid with a Tn10 transposon; (2) disrupt the chromosomal gene by *a*: introducing the Tn10 via plasmid recombination with the chromosomal gene, *b*: curing the cell of the plasmid, and *c*: selecting for tetracycline resistant colonies; (3) confirm the presence of the transposon in the gene, by *a*: isolating chromosomal DNA from the wild type and transformed cell; *b*: digesting it with a restriction enzyme (preferably one that doesn't cut the wild type gene); *c*: contruct a probe by labelling the cloned gene and incubate with the chromosomal DNA; *d*: run samples on an agarose gel and compare hybridization patterns; the gene disrupted with the transposon will show a different migration pattern from the nondisrupted gene; and (4) use the genetic assay system described to monitor VSP repair. If the Lac^+ reversion frequency increases significantly, the disrupted chromosomal gene is required for VSP repair; consequently, the cloned gene is required for VSP repair and complements *mutG*. However, if the Lac^+ reversion frequency remains low, the disrupted chromosomal gene is not required for VSP repair. Therefore, the cloned gene is not involved in VSP repair and may somehow suppress *mutG*. Deciphering this suppression mechanism would help our understanding of VSP repair.

A fragment which complements the mutation has been

cloned and partially sequenced. Further characterization of this cloned fragment - a complete nucleotide sequence and restriction enzyme map - is currently under way. Once the complete nucleotide sequence of the complementing fragment is determined, the identification of open reading frames, complementation testing, the comparison of the nucleotide sequence to characterized genes, and testing the activity of the purified gene product via *in vitro* heteroduplex experiments may provide useful information as to the protein product of *mutG*, and in turn, provide insight to the mechanism of VSP repair. Although there is still a lot of research left to do to answer the initial question, most of the ground work has been completed.

Additional experiments will be necessary to determine whether information gained by studying SPR112 will extend our understanding of the mechanism of VSP repair. So far, a strain deficient in a gene necessary for VSP repair has been isolated. The mutation has been mapped and experimental results show that the mutation is not in the *vsr* gene but in another gene; we've designated this mutator locus *mutG*. Even if it is vsr^-, it is the first such strain isolated and will be used to elucidate the methylation/deamination/ and repair interaction *in vivo*.

In human HeLa cells JIRICNY and coworkers have characterized a mismatch repair system in humans which, like VSP repair, corrects T·G mismatches arising from deamination of 5-methylcytosines (BROWN and JIRICNY, 1987). A thymine

glycosylase removes the deaminated base (thymine), generating an apyrimidinic (AP) site opposite the guanine (WIEBAUER and JIRICNY, 1989; WIEBAUER and JIRICNY, 1990). The single nucleotide gap, generated by the cleaving of the sugar-phosphate backbone by an AP endonuclease, is filled by DNA polymerase ß (WIEBAUER and JIRICNY, 1989; WIEBAUER and JIRICNY, 1990).

In *E. coli*, the very short patch repair system seems to process G·T mismatches that result from the deamination of 5-methylcytosines by a different process from that used in human cells. The protein product of gene *vsr* has been purified and characterized (HENNECKE *et al.*, 1991). Results of *in vitro* experiments propose that *vsr* codes for a DNA mismatch specific endonuclease; it only nicks the T-containing strand of G·T mismatches which result from the deamination of 5-methylcytosines. However, *in vivo* assays suggest the products of *mutL* and *mutS* are involved in VSP repair (LIEB, 1987). So *in vitro*, it appears *vsr* is sufficient for VSP repair; *in vivo*, more genes may be required for optimal VSP repair, one of which may be *mutG*.

Last but not least, "Why study DNA mismatch repair mechanisms in *E. coli*?" The basic chemistry of DNA is the same throughout nature. Studies of DNA repair have suggested that many of the same strategies are used by both prokaryotes and eukaryotes and by unicellular and multicellular organisms. Extrapolation from a well-studied organism, such as *E. coli*, to a less well-studied organism, such as humans

for example, can be a powerful aid in the analysis of DNA repair. Although the strategy used to repair G·T mismatches resulting from the spontaneous deamination of 5-methylcytosines in *E. coli* appears to be different from the one characterized in humans, more homology may exist at the protein level. Also, would JIRICNY have looked for DNA repair systems in humans had they not been found in *E. coli* or another prokaryote?

Determining the molecular mechanism of DNA repair should yield insight into other fundamental cellular processes and into the relationship between DNA repair and chemical carcinogenesis (WALKER *et al.*, 1985). The complex problems that must now be addressed invite the combined use of all experimental approaches available, including the genetic analysis of DNA repair (WALKER *et al.*, 1985).

BIBLIOGRAPHY

AKIYAMA, M., H. MAKI, M.SEKIGUCHI, and T. HORIUCHI 1989. A specific role of MutT protein: To prevent dG·dA mispairing in DNA replication. Proc. Natl. Acad. Sci. USA 86: 3949 - 3952.

AU, K.G., M. CABRERA, J.H. MILLER, and P. MODRICH 1988. *Escherichia coli* mutY gene product is required for specific A·G to C·G mismatch correction. Proc. Natl. Acad. Sci. USA 85: 9163 - 9166.

AU, K.G., S. CLARK, J.H. MILLER, and P. MODRICH 1989. *Escherichia coli mutY* gene encodes an adenine glycosylase active on G·A mispairs. Proc. Natl. Acad. Sci. USA 86: 8877 - 8881.

BIRNBOIM, H.C. and J. DOLY 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513.

BACHMANN, B.J. 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiological Reviews, 54 (2): 130 - 197.

BHATNAGAR, S.K., and M.J. BESSMAN 1988. Studies on the mutator gene *mutT* of *Escherichia coli*: Molecular cloning of the gene, purification of the gene product, and identification of a novel nucleoside triphosphatase. J. Biol. Chem. 263: 8953 - 8957.

BOITEUX, S., T.R. O'CONNER, F. LEDERER, A. GOUYETTE, and J. LAVAL 1990. Homogenous *Escherichia coli* FPG protein. J. Biol. Chem. 265: 3916 - 3922.

BROOKS, P., C. DOHET, G. ALMOUZNI, M. MECHALI, and M. RADMAN 1989. Mismatch repair involving localized DNA synthesis in extracts of *Xenopus* eggs. Proc. Natl. Acad. Sci. USA 86: 4424 - 4429.

BROWN, T.C., and J. JIRICNY 1987. A specific mismatch repair event protects mammalian cells from loss of 5-methylcytosine. Cell 50: 945 - 950.

CABRERA, M., Y. NGHIEM, and J.H. MILLER 1988. *MutM*, a second mutator locus in *Escherichia coli* that generates G·C to T·A transversions. J. Bacteriol. 170 (11): 5405 - 5407.

COHEN, S.N., A.C.Y. CHANG, and L. HSU 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69: 2110.

COULONDRE, C. and J.H. MILLER 1977. Genetic studies of the *lac* repressor. III. Additional correlation of mutational sites with specific amino acid residues. J. Mol. Biol. 117: 525 - 576.

COX, E.C. 1976. Bacterial mutator genes and the control of spontaneous mutation. Annu. Rev. Genet. 10: 135 - 156.

CLAVERYS, J-P., H. PRATS, H. VASSEGHI, and M. GHERARDI 1984. Identification of *Streptococcus pneumoniae* mismatch repair genes by an additive transformation approach. Mol. Gen. Genet. 196: 91 - 96.

CUPPLES, C.G. and J.H. MILLER 1988. Effects of amino acid substitutions at the active site in *Escherichia coli* ß-galactosidase. Genetics 120: 637 - 644.

CUPPLES, C.G. and J.H. MILLER 1989. A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions. Proc. Natl. Acad. Sci. USA 86: 5345 - 5349.

DAVIS, R.W., D. BOTSTEIN, and J.R. ROTH 1980. *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

DRAKE, J.W. 1969. Comparative rates of spontaneous mutation. Nature 221: 1132.

FREIFELDER, D.M. 1987. *Microbial Genetics*. Jones and Bartlett Publishers, University of California, San Diego.

GRILLEY, M., J. HOLMES, B. YASHAR, and P. MODRICH 1990. Mechanisms of DNA-mismatch correction. Mutation Research 236: 253 - 267.

HATTMAN, S., C. KENNY, L. BERGER, and K. PRATT 1978. Comparative study of DNA methylation in three unicellular eucaryotes. J. Bacteriol. 135: 1156 - 1157.

HENNECKE, F., H. KOLMAR, K. BRUNDL, and H-J. FRITZ 1991. The *vsr* gene product of *E. coli* K12 is a strand- and sequence-specific DNA mismatch endonuclease. Nature 353: 776 - 778.

HERMAN, G.E., and P. MODRICH 1981. *Escherichia coli* K12 clones that overproduce *dam*-methylase are hypermutable. J. Bacteriol. 145: 644 - 646.

HOLLIDAY, R. 1964. A mechanism for gene conversion in fungi. Genet. Res. 5: 282 - 304.

HOLMES, J., S. CLARK, and P. MODRICH 1990. Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. Proc. Natl. Acad. Sci. USA 87: 5837 - 5841.

ISH-HOROWICZ, D. and J.F. BURKE 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. 9: 2989.

INNIS, M.A., D.H. GELFAND, J.J. SNINSKY, and T.J. WHITE 1990. *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., San Diego, California.

JIRICNY, J., M. HUGHES, N. CORMAN, and B.B. RUDKIN 1988. A human 200-kDa protein binds selectively to DNA fragments containing G·T mismatches. Proc. Natl. Acad. Sci. USA 85: 8860 - 8864.

JONES, M., R. WAGNER, and M. RADMAN 1987a. Mismatch repair of deaminated 5-methyl-cytosine. J. Mol. Biol. 194: 155 - 159.

JONES, M., R. WAGNER, and M. RADMAN 1987b. Mismatch repair and recombination in *E. coli*. Cell 50: 621 - 626.

KLEINA, L.G., J.M. MASSON, J. NORMANLY, J. ABELSON, and J.H. MILLER 1990. Construction of *Escherichia coli* amber suppressor tRNA genes; II. Synthesis of additional tRNA genes and improvement of suppressor efficiency. J. Mol. Biol. 213: 705 - 717.

KOHARA, Y., K. AKIYAMA, and K.ISONO 1987. The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. Cell 50: 495 - 508.

KRAMER, B., W. KRAMER, and H-J. FRITZ 1984. Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. Cell 38: 879 - 887.

LANGLE-ROUAULT, F., G. MAENHAUT-MICHEL, and M. RADMAN 1987. GATC sequences, DNA nicks and the mutH function in *Escherichia coli* mismatch repair. The EMBO Journal 6: 1121 - 1127.

LANGRIDGE, J. 1974. Mutation spectra and the neutrality of mutations. Aust. J. Biol. Sci. 27: 309 - 320.

LANGRIDGE, J., and J.H. CAMPBELL 1969. Classification and intragenic position of mutations in the ß-galactosidase gene of *Escherichia coli*. Mol. Gen. Genet. 103: 339 - 347.

LEATHERBARROW, R.J., and A.R. FERSHT 1986. REVIEW: Protein engineering. Protein Engineering 1: 7 - 16.

LEWIN, B. 1987. *Genes III*. John Wiley and Sons, Inc. New York, USA.

LIEB, M. 1983. Specific mismatch correction in bacteriophage lamda crosses by very short patch repair. Mol. Gen. Genet. 191: 118 - 125.

LIEB, M. 1985. Recombination in the lamda repressor gene: Evidence that very short patch (VSP) mismatch correction restores a specific sequence. Mol. Gen. Genet. 199: 465 - 470.

LIEB, M. 1987. Bacterial genes *mutL*, *mutS*, and *dcm* participate in repair of mismatches at 5-methylcytosine sites. J. Bacteriol. 169: 5241 - 5246.

LIEB, M. 1991. Spontaneous mutation at a 5-methylcytosine hotspot is prevented by very short patch (VSP) mismatch repair. Genetics 128: 23 - 27.

LOECHLER, E.L., C.L. GREEN, and J.M. ESSIGMAN 1984. *In vivo* mutagenesis by O^6-methylguanine built into a unique site in a viral genome. Proc. Natl. Acad. Sci. USA 81: 6271 - 6275.

LOVELESS, A. 1969. possible relevance of O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature 223: 206 - 207.

LU, A-L., S. CLARK, and P. MODRICH 1983. Methyl-directed repair of DNA base-pair mismatches *in vitro*. Proc. Natl. Acad. Sci. USA 80: 4639 - 4643.

MAKI, H, and M. SEKIGUCHI 1992. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature 355: 273 - 275.

MANKOVICH, J.A., C.A. McINTYRE, and G.C. WALKER 1989. Nucleotide sequence of the *Salmonella typhimurium mutL* gene required for mismatch repair: homology of MutL to HexB of *Streptococcus pneumoniae* and to PMS1 of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 171 (10): 5325 - 5331.

MARINUS, M.G. 1984. Methylation of prokaryotic DNA, in A. RAZIN, H. CEDAR, and A.D. RIGGS (Eds.), DNA Methylation, Biochemistry and Biological Significance, Springer, New York, pp. 81 - 109.

MASSON, J.M., and J.H. MILLER 1986. Expression of synthetic tRNA genes under the control of a synthetic promoter. Gene 47: 179 - 183.

MICHAELS, M.L., C. CRUZ, and J.H. MILLER 1990. *mutA* and *mutC*: Two mutator loci in *Escherichia coli* that stimulate transversions. Proc. Natl. Acad. Sci. USA 87: 9211 - 9215.

MILLER, J.H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

MILLER, J.H. 1983. Mutational specificity in bacteria. Ann. Rev. Genet. 17: 215 - 238.

MODRICH, P. 1987. DNA mismatch correction. Ann. Rev. Biochem. 56: 435 - 466.

NGHIEM, Y., M. CABRERA, C.G. CUPPLES, and J.H. MILLER 1988. The *mutY* gene: A mutator locus in *Escherichia coli* that generates G·C to T·A transversions. Proc. Natl. Acad. Sci. USA 85: 2709 - 2713.

NORMANLY, J, J.M. MASON, L.G. KLIENA, J. ABELSON, and J.H. MILLER 1986. Construction of two *Escherichia coli* amber suppressor genes: tRNA (Phe CUA) and tRNA (Cys CUA). Proc. Natl. Acad. Sci. USA 83: 6548 - 6552.

PARKER, B. and M.G. MARINUS 1988. Gene 73: 531 - 535.

PROFFITT, J., J. DAVIE, D. SWINTON, and S. HATTMAN 1984. 5-Methylcytosine is not detectable in *Saccharomyces cerevisiae* DNA. Mol. Cell. Biol. 4: 985 - 988.

PRUDHOMME, M., B. MARTIN, V. MEJEAN, and J-P. CLAVERYS 1989. Nucleotide sequence of the *Streptococcus pneumoniae hexB* mismatch repair gene: homology of HexB to MutL of *Salmonella typhimurium* and to PMS1 of *Saccharomyces cerevisiae*. J. Bacteriol. 171 (10): 5332 - 5338.

PUKKILA, P.J., J. PETERSON, G. HERMAN, P. MODRICH, and M. MESELSON 1983. Effects of high levels of DNA adenine methylation on methyl-directed mismatch repair in *Escherichia coli*. Genetics 104: 571 - 582.

RADICELLA, J.P., E.A. CLARK, and M.S. FOX 1988. Some mismatch repair activities in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 85: 9674 - 9678.

RADMAN, M. and R. WAGNER. 1986. Mismatch repair in *Escherichia coli*. Ann. Rev. Genet. 20: 523 - 538.

RAPOSA, S., and M.S. FOX 1987. Some features of base pair mismatch and heterology repair in *Escherichia coli*. Genetics 117: 381 - 390.

ROSE, R.E. 1988. The Nucleotide Sequence of pACYC184. Nucleic Acids Res. 16: 355.

SAMBROOK, J., E.F. FRITSCH, and T. MANIATIS 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

SCHAAPER, R.M. and R.L. DUNN 1987. Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: The nature of *in vivo* DNA replication errors. Proc. Natl. Acad. Sci. USA 84: 6220 - 6224.

SCHAAPER, R.M., and M. RADMAN 1989. The extreme mutator effect of *Escherichia coli mutD5* results from saturation of mismatch repair by excessive DNA replication errors. EMBO Journal 8 (11): 3511 - 3516.

SINGER, M., T.A. BAKER, G. SCHNITZLER, S.M. DIESCHEL, M. GOEL, W. DOVE, K.J. JAACKS, A.D. GROSSMAN, J.W. ERICKSON, and C.A. GROSS 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. 53: 1 - 24.

SILHAVY, T.J., M.L. BERMAN, and L.W. ENQUIST 1984. Experiments with Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

SOHAIL, A., M. Lieb, M. DAR, and A.S. BHAGWAT 1990. A gene required for very-short-patch repair in *Escherichia coli* is adjacent to the DNA cytosine methylase gene. J. Bacteriol. 172 (8): 4214 - 4221.

SU, S-S., R. S. LAHUE, K. G. AU, and P. MODRICH 1988. Mispair specificity of methyl-directed DNA mismatch correction *in vitro*. J. Biol. Chem. 263 (14): 6829 - 6835.

TCHOU, J., H. KASAI, S. SHIBUTANI, M.-H. CHUNG, J. LAVAL, A.P. GROLLMAN, and S. NISHIMURA 1991. 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. Proc. Natl. Acad. Sci. USA 88: 4690 - 4694.

THOMAS, D.C., J.D. ROBERTS, and T.A. KUNKEL 1991. Heteroduplex repair in extracts of human HeLa cells. J. Biol. Chem. 266 (6): 3744 - 3751.

VILLEE, C.A., E.P. SOLOMON, and P.W. DAVIS 1985. *Biology*. Saunders College Publishing, New York, New York.

WALKER, G.C., L. MARSH, and L.A. DODSON 1985. Genetic analysis of DNA repair: Inference and extrapolation. Ann. Rev. Genet. 19: 103 - 126.

WELSH, K.M., A-L. LU, S. CLARK, and P. MODRICH 1987. Isolation and Characterization of the *Escherichia coli mutH* gene product. J. Biol. Chem. 262 (32): 15624 - 15629.

WHORISKEY, S.K., V-H. NGHIEM, P-M. LEONG, J.M. MASSON, and J.H. MILLER 1987. Genetic rearrangements and gene amplification in *Escherichia coli*: DNA sequences at the junctures of amplified gene fusions. Genes and Development 1: 227 - 237.

WIEBAUER, K., and J. JIRICNY 1989. *In vitro* correction of G·T mispairs to G·C pairs in nuclear extracts from human cells. Nature 339: 234 - 236.

WIEBAUER, K., and J. JIRICNY 1990. Mismatch-specific thymine DNA glycosylase and DNA polymerase ß mediate the correction of G·T mispairs in nuclear extracts from human cells. Proc. Natl. Acad. Sci. USA 87: 5842 - 5845.

YANOFSKY, C., E.C. COX, V. HORN 1966. The unusual mutagenic specificity of an *E. coli* mutator gene. Proc. Natl. Acad. Sci. USA 55: 274 - 281.

YEH, Y-C., D-Y. CHANG, J. MASIN, and A-L. LU 1991. Two nicking enzyme systems specific for mismatch-containing DNA in nuclear extracts from human cells. J. Biol. Chem. 266 (10): 6480 - 6484.

ZELL, R., and H-J. FRITZ 1987. DNA mismatch-repair in *Escherichia coli* counteracting the hydrolytic deamination of 5-methyl-cytosine residues. EMBO Journal 6 (6): 1809 - 1815.

APPENDICES

APPENDIX A: MEDIA

The following is a complete list, in alphabetical order, of the media used. Recipes are as described in MILLER (1972) and SAMBROOK (SAMBROOK *et al.*, 1982). Deionized distilled water was added until the final volume was reached. All media were sterilized by autoclaving for 30 minutes at 121°C, 15 lb/in.^{2} on the liquid cycle and were cooled to 55°C prior to pouring into petri dishes or adding supplements. For the complete list of supplements used, refer to ***APPENDIX B: MEDIA SUPPLEMENTS***.

CHLORAMPHENICOL PLATES

Per litre:

1 M NaOH	2.0 ml
Tryptone	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	0.5 g
H_2O	700.0 ml

Using 1 M NaOH, the pH was adjusted to 7.0. Then 10.0 grams of agar followed by distilled water were added, making a final 1 liter volume. Once sterilized 10 ml of sterile 20% glucose solution and 0.5 ml of 25 mg/ml of chloramphenicol (dissolved in 95% ethanol) were added to the medium.

LB MEDIUM

Per liter:

Bacto-Tryptone	10 g
Bacto-Yeast Extract	5 g
Sodium Chloride	10 g

100 ml aliquots were distributed to 200 ml bottles and the media was sterilized.

LB PLATES were made by adding 10 grams of agar to the ***LB MEDIUM*** ingredients. The medium was autoclaved and then cooled before being poured into petri dishes.

LB TOP AGAR

Per liter:

Bacto-Tryptone	10 g
Bacto-Yeast Extract	5 g
Sodium Chloride	10 g
Agar	5 g

The agar was dissolved by heating the solution on a hot plate/stirrer. Once completely dissolved, 100 ml aliquots were distributed into 200 ml bottles and were sterilized.

MINIMAL GLUCOSE LIQUID MEDIUM

All the solutions used to make minimal glucose medium were sterile.

To make 100 ml:

Distilled Water	89 ml
10X Min A*	10 ml
20% Glucose	1 ml
20% $MgSO_4$	100 µl
1% Thiamine	50 µl

* For the composition of 10X Min A solution, refer to ***APPENDIX C: BUFFERS***.

MINIMAL GLUCOSE PLATES

The following sterile solutions:

20% Glucose	10 ml
20% $MgSO_4$	1 ml
1% Thiamine	0.5 ml

were added to 100 ml of sterile 10X minimal A solution (for composition of buffer, refer to ***APPENDIX C: BUFFERS***). 10 g of agar were dissolved in 900 ml of distilled water and the medium was sterilized. Once cooled, the supplemented 10X minimal A solution was added and mixed. Plates were poured.

MINIMAL LACTOSE PLATES

The composition is identical to the minimal glucose plates, described above, with the exception that 20% lactose solution was used instead of the 20% glucose solution.

NZCYM MEDIUM

Per litre:

To 950 ml of deionized H_2O, add:

NZ Amine	10 g
NaCl	5 g
Yeast Extract	5 g
Casamino Acids	1 g
$MgSO_4 \cdot 7H_2O$	2 g

Solution was dispensed in 100 ml aliquotes in 200 ml bottles. Medium was autoclaved.

SUPERBROTH

Per 100 ml:

Tryptone	3.2 g
Yeast Extract	2.0 g
Sodium Chloride	0.5 g
1 M NaOH	0.5 ml

APPENIX B: MEDIA SUPPLEMENTS

SUPPLEMENT	CONCENTRATION	STERILIZATION TECHNIQUE	FINAL CONC.
Ampicillin	100 mg/ml H_2O	filter	100 mg/L
Chloramphenicol	20 mg/ml EtOH	----	20 mg/L
IPTG	100 mg/ml H_2O	filter	100 mg/L
Kanamycin	50 mg/ml H_2O	filter	50 mg/L
Methionine	10 mg/ml H_2O	autoclave	50 mg/L
Naladixic acid	100 mg/ml 1M NaOH	----	30 mg/L
Proline	10 mg/ml H_2O	autoclave	100 mg/L
P-gal	50 mg/ml H_2O	autoclave	500 mg/L
Rifampicin	50 mg/ml methanol	----	100 mg/L
Streptomycin	100 mg/ml H_2O	filter	100 mg/L
Tetracycline	15 mg/ml 50% EtOH	----	15 mg/L
X-gal	20 mg/ml formamide	----	40 mg/L

[a]Filter sterilization involves passing the solution through a 0.22-micron filter.

[b]All antibiotics, IPTG, and X-gal stock solutions are stored at -20°C.

[c]Methionine, proline, and P-gal stock solutions are stored at room temperature.

APPENDIX C: BUFFERS

Buffer recipes are as described in MILLER (1972) and SAMBROOK (SAMBROOK *et al.*, 1982). For all buffers, deionized distilled water was added until the final volume was reached. Sterilization was done by autoclaving for 30 minutes at 15 lb/in.^{2} on liquid cycle.

10X MINIMAL A SOLUTION

Per liter:

Potassium Phosphate Dibasic	105 g
Potassium Phosphate Monobasic	45 g
Ammonium Sulphate	10 g
Sodium Citrate·$2H_2O$	5 g

100 ml aliquots were distributed into 200 ml bottles and were sterilized.

SM SOLUTION: LAMDA STORAGE BUFFER

Per liter:

NaCl	5.8 g
$MgSO_4 \cdot 7H_2O$	2.0 g
1 M Tris·Cl (pH 7.5)	50.0 ml
2% Gelatin	5.0 ml

10X TBE (REGULAR)

Per liter:

Tris Base	108.0 g
Boric Acid	55.0 g
0.5 M EDTA (pH 8.0)	40.0 ml

10X TBE (SEQUENCING GRADE)

Per liter:

Tris Base	162.0 g
Boric Acid	50.0 g
EDTA	7.5 g

were dissolved in deionized distilled water. The pH was adjusted to 8.8.

APPENDIX D: SOLUTIONS FOR RAPID PLASMID PREPARATION

The composition of the three solutions used for rapid plasmid preparations is from the methods of BIRNBOIM and DOLY (1979) and ISH-HOROWICZ and BURKE (1981). Only deionized distilled water was used.

***SOLUTION I*:**

To make a 100 ml:

40% Glucose	2.26 ml
1 M Tris·Cl (pH 8.0)	2.5 ml
1 M EDTA (pH 8.0)	1.0 ml
H_2O	94.25 ml

Solution I was sterilzed by autoclaving for 25 minutes at 15 lb/in.^{2} on liquid cycle.

SOLUTION II

This solution was made fresh before use.

To make 10 ml:

1 N Sodium Hydroxide	2.0 ml
10% SDS*	1.0 ml
H_2O	7.0 ml

*Sodium Dodecyl Sulphate

SOLUTION III

To make 100 ml:

5 M potassium acetate	60.0 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

PH was adjusted to 4.8.

APPENDIX E: COMMONLY USED EQUIPMENT

EQUIPMENT Make (discription)	Model
CLINICAL CENTRIFUGE: International Equipment Co.	HN-SII
BENCHTOP MICROFUGE: Canlab (Biofuge A)	1302
CENTRIFUGE: Beckman (Induction Drive) (rotor: JA17)	J2-21M
ULTRACENTRIFUGE: International Equipment Co.	B-20A
LYOPHILIZER Savant (Speed Vac Concentrator)	SVC100H-115
INCUBATOR John's Scientific	1540
SHAKER INCUBATOR New Brunswick Scientific Co.	G25
SPECTROPHOTOMETER LKB (Biochrom Ultro Spec III)	4050