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**Shall We Dance? Investigating Protein Interactions Implicated
in *Escherichia coli* Postreplicative Mismatch Repair
Using a Bacterial Two Hybrid System**

Catherine A. Mansour

A Thesis

in

The Department

of

Biology

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ABSTRACT

Shall We Dance? Investigating Protein Interactions Implicated in *Escherichia coli* Postreplicative Mismatch Repair Using a Bacterial Two Hybrid System

Catherine A. Mansour

There are many potential mutational threats to the integrity of DNA and multiple DNA repair systems to counteract these. MutS, MutL and MutH are integral elements of *dam*-directed repair (MMR), a mismatch recognition system involved in rectifying DNA replication errors. Moreover, MutS and MutL have been implicated in the effective endonucleolytic functioning of Vsr, primary player of very short patch (VSP) repair. This study made use of the bacterial two hybrid system in an attempt to further clarify the critical role that each of the above-mentioned proteins plays relative to one another.

Our initial goal was to determine specific interactions involved in the MMR pathway. Of the six positive candidates first revealed, four known interactions were observed. A novel liaison between MutS and MutH was detected while binding of MutL to Vsr established a direct link between MMR and VSP repair. The disappearance of the MutS-MutH complex in a *mutL* background makes a strong case for the role of MutL as a molecular matchmaker. The presence of Vsr impeded proper function of MMR as demonstrated by the lack of MutL homodimerization as well as the disappearance of all interactions involving MutL. This evidence lends further credence to MutL's role as coordinator of MMR and VSP repair.

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The Peanut Gallery, most especially I. M. (x), Cousin It, Shaggy and the Captain, among others (you know who you are !), for making me realize that one does not “discover new land without consenting to lose sight of the shore for a very long time.” For your constant support, laughter and help in so many ways that it would take too long to list here.....thanks !!

Press on. Nothing in the world can take the place of persistence. Talent will not; nothing in the world is more common than unsuccessful men with talent. Genius will not; unrewarded genius is a proverb. Education will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent...

-Calvin Coolidge

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1.0 Introduction

1.1 Maintenance of Genomic Integrity and the Need for Repair Mechanisms

All living things, great or small, possess one common feature that inexorably links them to each other and transcends all organismal barriers. Each harbours genetic material containing all the necessary information to ensure survival. Damage to DNA can result in the loss of genetic information and can lead to genomic instability, mutations and cell death. Organisms are provided with multiple DNA damage repair systems, which differ in the type of damage they can repair as well as in the way they function. The importance of such systems being in place can be understood by simply examining the extensive and diversified list of potential damage that can be incurred by genetic material (Figure 1).

1.2. Mismatch Repair Systems

1.2.1 Short Patch Repair

Any one of the nitrogenous bases can undergo spontaneous alterations. Such modifications can include tautomeric shifts as well as the loss or deamination of bases (Friedberg *et al.*, 1995). The latter change involves the loss of an amino group. One example is the conversion of 5-methylcytosine, an analog of cytosine, to thymine, a normal constituent of DNA (Figure 2). Small quantities of this analogue have been found scattered throughout the *E. coli* genome (Lindahl, 1979) as well as in some eukaryotes (Ehrlich *et al.*, 1990).

I Spontaneous Alterations of and Damage to DNA

A. Mismatches

B. Spontaneous Alterations in the Chemistry of DNA Bases

1. Tautomeric Shifts

2. Deamination of Bases

- Deamination of Cytosine
- Incorporation of Uracil into DNA during Semiconservative Replication
- Deamination of Cytosine Analogs
- Deamination of Adenine and Guanine

3. Loss of Bases-Depurination and Depyrimidination

4. Oxidative Damage

II Environmental Damage to DNA

A. Physical Agents That Damage DNA

1. Ionizing Radiation

- Base Damage
- Sugar Damage & Strand Breaks

2. UV Radiation

- Cyclobutane Pyrimidine Dimers
- Pyrimidine-Pyrimidone (6-4) Photoproducts
- Spore photoproducts
- Complex Lesions Involving Purines
- Pyrimidine hydrates
- Thymine Glycol
- DNA Cross-Links and Strand Breaks
- Photosensitization

B. Chemical Agents That Damage DNA

- Alkylating Agents
- Cross-Linking Agents
- N-2-Acetyl-2-Aminofluorene
- Benzo[a]pyrene
- Aflatoxins
- N-Methyl-N'-Nitro-N-Nitrosoguanidine

Fig. 1. Types of DNA Damage. A diverse listing of possible modifications to the molecular structure of genetic material.

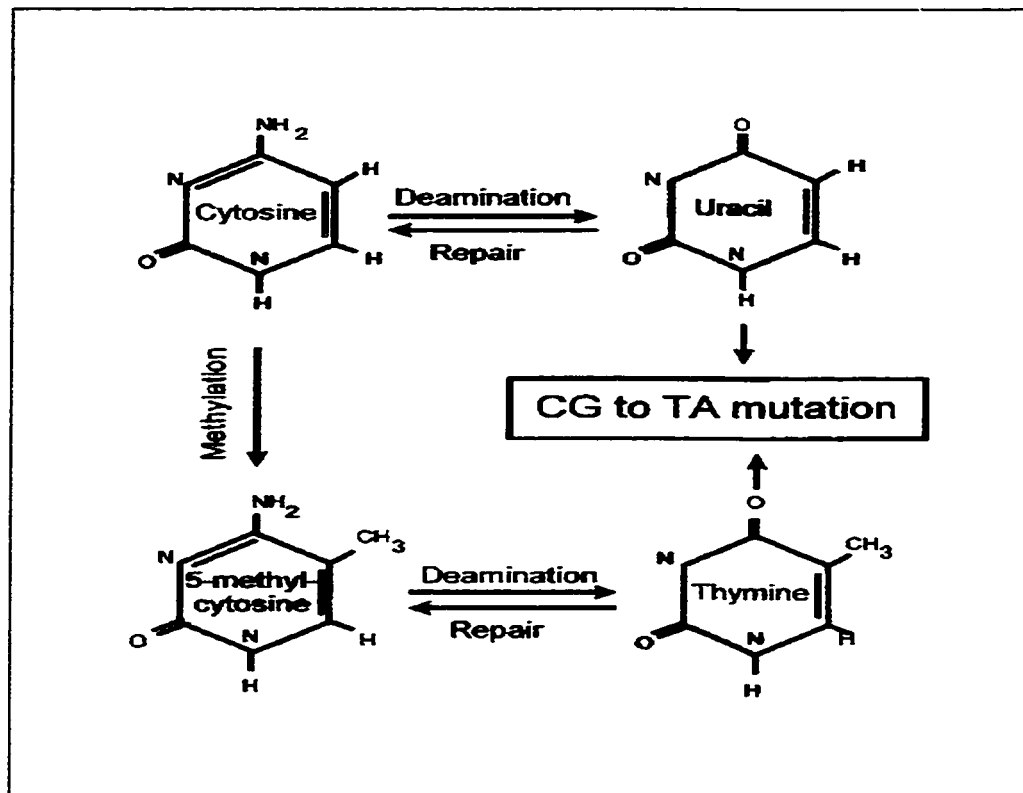


Fig. 2. Deamination of 5-methylcytosine in DNA. Methylation of cytosine at the 5-position by Dcm (DNA cytosine methylase) gives rise to 5-methylcytosine. Spontaneous hydrolytic deamination involves the loss of an amino group. Such modification of this analogue leads to the appearance of a thymine. If left unrepaired, this lesion leads to a CG to TA transition mutation following the next round of replication.

In *Escherichia coli*, the internal cytosine of the pentanucleotide stretch of DNA CC(A/T)GG is methylated at the 5-position by DNA cytosine methyltransferase (May & Hattman, 1975), product of the *dcm* gene (Marinus, 1973). The 5-methylcytosines present in Dcm methylation recognition sites are prone to spontaneous deamination, resulting in a T:G mispair (Coulondre *et al.*, 1978). If left unrepaired, a C:G to T:A transition mutation will arise following the next round of DNA replication. Removal of the offending pyrimidine and its replacement by the proper base is the responsibility of the very short patch repair (VSPR) system.

The first indication that such a system existed was given when it was observed that recombination frequencies of certain amber mutations in the repressor (*cI*) gene of bacteriophage λ were higher than predicted by the physical distance separating them (Lieb, 1981). Further study of one of these mutations, *am6*, presented evidence that this anomaly arose from a C \rightarrow T mutation developing in a glutamine codon located within the Dcm recognition sequence CCAGG (Lieb, 1983). This same study demonstrated that the excess number of *cI*⁺ recombinants could be attributed to the existence of a system repairing mutations as close as 10 nucleotides but not in areas further than 20 base pairs. This characteristic is reflected in the repair system's name. It was later shown that this short patch repair system does indeed maintain the CCAGG sequence, in addition to other related sequences, as demonstrated by the preferential repair of the *am6* sequence (CTAGG) to the *cI*⁺ (CCAGG) sequence (Lieb, 1985; Lieb *et al.*, 1986). Such repair thus restores the original C:G base pairing.

Assorted players involved in VSP repair include Vsr (*vsr*) (Sohail *et al.*, 1990) and DNA polymerase I (*polA*) (Dzidic & Radman, 1989). The crystal structure of Vsr has recently been elucidated (Tsutakawa *et al.*, 1999). Interestingly, the MMR genes *mutL* and *mutS* are also involved in the maintenance of this repair system's efficiency (Lieb, 1987). Disabling either gene leads to a decrease in the excess number of wild type *cl* recombinants, a benchmark associated with the presence of VSP repair. Such a decrease was not observed in *mutH* or *uvrD* strains. An even greater decrease in *cl*⁺ recombinants was observed upon the introduction of a *dcm* mutation. It is interesting to note that the decrease in VSPR efficiency attributed to lacking MutS and/or MutL can be counteracted by the introduction of a plasmid containing *vsr* (Sohail *et al.*, 1990)

Sohail *et al* (1990) established that Dcm is not necessary for the proper functioning of VSP repair. This is an unusual paradox since it is the presence of the methyltransferase that indirectly provides the lesions which Vsr repairs. Both *dcm* and *vsr* find themselves in a unique genetic arrangement (Figure 3). The 5' end of *vsr* overlaps the 3' end of *dcm* by 6 codons in a +1 reading frame (Sohail *et al.*, 1990). In addition, although these genes are transcribed from the same promoter, two separate proteins are produced (Sohail *et al.*, 1990).

VSP repair is initiated by the 18 kDa product of *vsr*, located at 43 minutes on the *E. coli* chromosome (Sohail *et al.*, 1990). This 156 amino acid protein functions as a strand-specific endonuclease (Hennecke *et al.*, 1991). The efficiency with which it recognizes and cleaves T/G mismatches is dependant upon the tetranucleotide

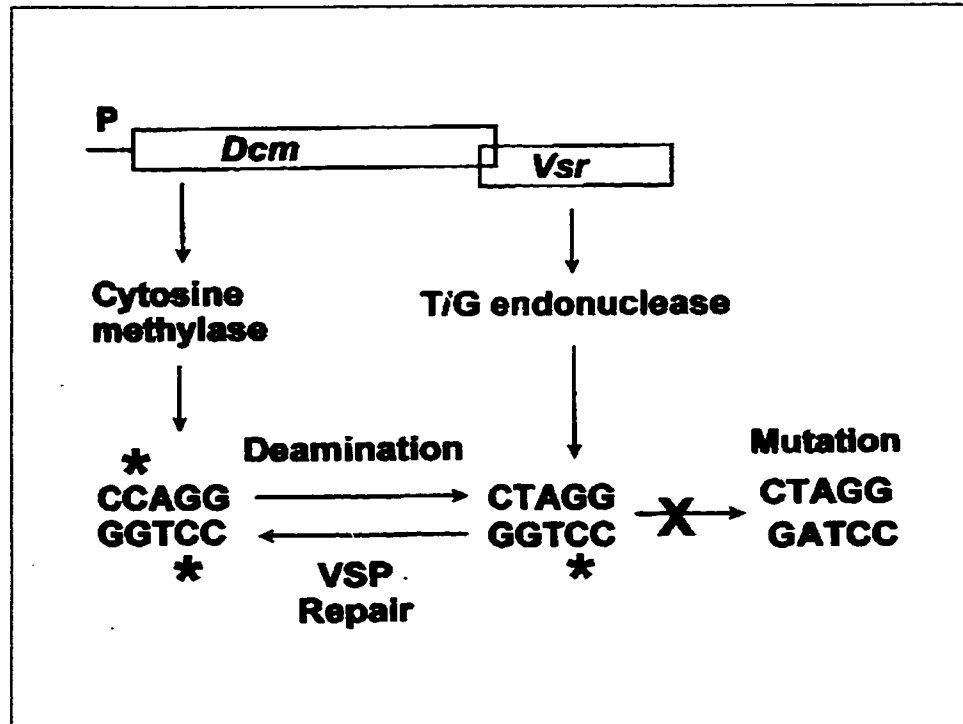


Fig. 3. The *dcm-vsr* operon. *dcm* and *vsr* are found in a unique genetic arrangement. This operon is organized such that the 5' end of *vsr* overlaps the 3' end of *dcm* by 6 codons in a +1 reading frame. The 5-methylcytosine present within the *Dcm* recognition site is prone to spontaneous deamination. Maintenance of the original sequence is achieved by the removal of the resulting thymine and is the responsibility of very short patch repair.

sequence context within which the mismatch is located (Lieb & Bhagwat, 1996). Endonucleolytic attack by Vsr is aimed directly 5' to the unwanted thymine. DNA polymerase I, with its inherent 5' → 3' exonuclease activity, then proceeds with the removal of the pyrimidine. DNA ligase seals the nick following DNA resynthesis.

Several reasons have been presented to explain the *raison d'être* of very short patch repair. Zell and Fritz (1987) proposed that VSPR functions in the reduction of the mutation level associated with spontaneous deamination of 5-methylcytosines. This postulate was corroborated by Lieb (1991). In lysogens where only Dcm was present, a drastic increase in such mutations was observed at a site known to be a hot spot for these. This hot spot disappeared when both Dcm and Vsr were removed while the addition of Vsr alone caused a four-fold decrease in mutation frequency. Evidence that VSPR is crucial to the maintenance of the Dcm recognition sequence as well as sequences that are similar but not recognized by the cytosine methyltransferase has already been brought to light (Lieb, 1987). Based on this, Bhagwat and McClelland (1992) proposed that VSP might have a hand in shaping the composition of the genome. They determined that tetranucleotide sequences containing thymine such as CTAG tend to be under-represented while those sequences containing cytosines (CCAG) were present in larger amounts.

An analog of cytosine, 5-methylcytosine (5-MeC), exists in most eukaryotes. The presence of 5-MeC has been implicated in the regulation of gene expression among other functions (Colot & Rossignol, 1999). Cytosine methylation usually takes place at Cp·G dinucleotides. Deamination of this nucleotide does transpire and

brings with it the implication that a system functioning in the repair of these lesions must be present. Very little has been discovered relative to the possible repair mechanism employed by eukaryotes for this type of lesion. Specific restoration of Cp-G pairs in primate cells (Brown & Jiricny, 1987) and chicken embryos (Jost *et al.*, 1995) was shown to occur following initial deamination of 5-MeC to thymine. Nedderman and Jiricny (1993) isolated an enzyme in human cells which excises thymine from a T/G mismatch. Characterization revealed it to be a glycosylase with no endonucleolytic activity. Interestingly, the use of glycosylases as the enzymes of choice for removal of T/G mismatches has been observed in Archaeobacteria (Yang *et al.*, 2000; Begley & Cunningham, 1999; Horst and Fritz, 1996). Short patch repair activity was very recently demonstrated in *Saccharomyces cerevisiae* although it was not directed towards the repair of mismatches arising due to deamination of 5-MeC (Coïc *et al.*, 2000).

1.2.2 Long Patch Repair

Mispairing of nucleotides and misalignment of the two complementary DNA strands are examples of spontaneous alteration. The latter is known as insertion/deletion loops (IDLs) while the former leads to the disruption of normal Watson-Crick pairings. Such mismatches can arise during DNA synthesis, DNA recombination or as a consequent to the chemical modification (*ie.* deamination) of a base (Marra & Schär, 1999). Mismatches that occur during DNA replication arise due to the error rate inherent to this process. Error frequencies are typically only 10^{-9}

to 10^{-10} per base replicated (Cox, 1976). Although not the only strategies available to the cell, there exist various levels of protection, each reinforcing the action of the previous, to ensure the maintenance of DNA replication fidelity. DNA polymerase III is responsible for proper base selection and insertion (Welsh & McHenry, 1982). In addition, it possesses proofreading activity responsible for the elimination of incorrectly inserted nucleotides (Schuermann & Echols, 1984). If replication errors or strand slippages escape the proofreading net, methyl-directed mismatch repair (MMR) can recognize and eliminate them.

MMR in *Escherichia coli* is responsible for the identification and removal of base-base mismatches (Modrich, 1991), insertion/deletion loops of less than four nucleotides (Dohet *et al.*, 1986; Parker & Marinus, 1992) as well as homologous recombination intermediates (Radman & Wagner, 1986; Worth *et al.*, 1994). It was later proven that IDLs of up to 4 or 5 bases can be repaired, albeit at lower efficiencies than those containing 1 to 3 bases (Fang *et al.*, 1997). Evidence of the existence of mismatch repair in prokaryotes and the idea of strand discrimination originated from early work involving transformation studies in *Streptococcus pneumoniae* (Claverys & Lacks, 1986). In a *S. pneumoniae* wild type strain, donor markers are integrated into the host genome at differing efficiencies. Mismatch repair was proposed to be responsible for this phenomenon. The heteroduplexes formed during the integration contain mismatches since the regions involved are similar but not identical. It appeared that the transformation efficiency was dependent not only upon the system's preference for undertaking repair on the donor strand but also on

its preference for the type of mismatch to repair. Strand discrimination was taken care of by recognizing that the donor strand possessed strand breaks at its ends. This idea was firmly established following the isolation of certain *hex*⁻ mutants (integrated all markers at equal frequency) found to be defective in MMR.

Methyl-directed mismatch repair in *E. coli* was believed to function in the same manner. Corroboration for this was two-fold. MutS from *Salmonella typhimurium* is homologous to HexA from *Streptococcus pneumoniae*. The former has been found to possess the ability to complement an *Escherichia coli* strain deficient in MutS, the recognition element of methyl-directed mismatch repair (Haber *et al.*, 1988). Strand discrimination is required in order to direct repair to the correct strand. (Lahue *et al.*, 1986). The idea of strand discrimination based on adenine methylation of d(GATC) sites was proposed by Wagner and Meselson (1976). The need for hemimethylated d(GATC) sites was later established (Lahue *et al.*, 1987). In essence, the window of opportunity for MMR to act lies in its ability to distinguish newly-synthesized DNA from parental DNA. This is achieved by virtue of the fact that, after synthesis, the d(GATC) sites of the nascent DNA strand are undermethylated as compared to those of the parental strand. Methylation of the adenine in the d(GATC) sites is entrusted to the DNA adenine methylase (*dam*). The importance of methylation was confirmed by the observation that *dam* strains of *E. coli* have increased rates of spontaneous mutation (Marinus & Morris, 1973, 1975). Another feature of MMR is its ability to act on a mismatch despite the nearest hemimethylated site being located up to two kilobases away (Lu *et al.*, 1986).

Removal and resynthesis of DNA spanning up to three kilobases has been previously observed (Wagner & Meselson, 1976). This is in sharp contrast to the short repair tracts witnessed in another base-specific mismatch repair system, very short patch repair (VSPR).

The presence of the *mutS*, *mutL* and *mutH* gene products, as well as DNA helicase II (*uvrD*), single-strand DNA binding protein (SSB), DNA polymerase III holoenzyme, exonuclease I, exonuclease VII, RecJ and DNA ligase (Lahue *et al.*, 1989) is required for the proper functioning of MMR. The *mutS* gene codes for an 853 amino acid protein (Schlensong & Böck, 1991) with a monomer molecular mass of approximately 97 kDa (Su *et al.*, 1988). A MutS homodimer acts as the recognition component of MMR by distinguishing base-base mispairs (Su & Modrich, 1986) and insertion/deletion loops (IDLs) (Parker & Marinus, 1992). Binding of MutS to mismatches contained in stretches of up to 20 nucleotides was first demonstrated by DnaseI footprint analysis (Su & Modrich, 1986). MutS has varying affinities for the eight possible mispairs where a T/G mismatch is the most efficiently repaired followed closely behind by the C/A mismatch (Su & Modrich, 1986; Su *et al.*, 1988). G/G and A/A mispairs are efficiently corrected while the repair efficiency of T/T, C/T and G/A mispairs was deemed to be of an intermediate nature (Kramer *et al.*, 1984). The remaining C/C mismatch, although recognized by MutS (Su *et al.*, 1988), does not appear to be subject to repair. Although mispairs leading to transition mutations are for the most part better repaired than those leading to transversion mutations, increased efficiency of repair may be dependent upon

sequence context. More specifically, an increase of G·C content in the surrounding area may be of influence (Jones *et al.*, 1987).

MutS, which demonstrates a weak ATPase activity (Haber & Walker, 1991), binds to the mismatch thus initiating repair (Su and Modrich, 1986) (Figure 4). Proper functioning of this enzyme has been attributed to the essential need for ATP hydrolysis (Au *et al.*, 1992). Introduction of mutations in the P-loop motif of the MutS ATP-binding site results in a dominant negative mutator phenotype and underscores the importance of its ATPase activity (Wu & Marinus, 1994). MutS, a mismatch and adenosine triphosphate are enough to catalyze the creation of an α -shaped DNA loop within which the mismatch is located (Allen *et al.*, 1997). Mut L, a 76 kDa homodimer, joins itself to the MutS-DNA complex in an ATP-dependent reaction (Grilley *et al.*, 1989) Subsequent translocation of the whole complex along the DNA ensues (Allen *et al.*, 1997).

The presence of MutL, a Mg^{2+} -dependent ATPase (Ban *et al.*, 1999) which catalyzes the hydrolysis of ATP more slowly than other ATPases (Ban and Yang, 1998), contributes to the increase in size of the α -loops with time (Allen *et al.*, 1997). Crystal structure studies revealed the independent binding of one ATP to each MutL subunit (Ban *et al.*, 1999). Dimerization requires the ordering of five loops located along the common boundary between subunits. This event takes place consequent to ATP binding. Once the subunits are joined, ATP hydrolysis can be carried out. In summary, activation of MutL occurs in the following order : 1.ATP binding - 2.loop ordering - 3.dimerization - 4.ATP hydrolysis. Some structural similarity exists

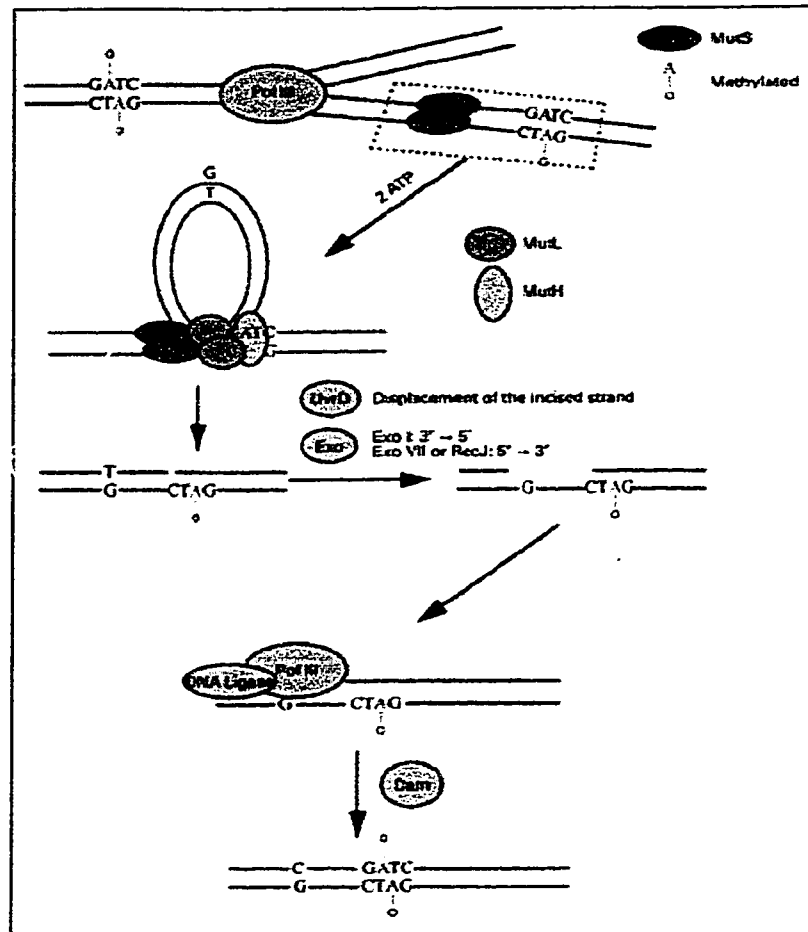


Fig. 4. Model for the mechanism of methyl-directed mismatch repair. DNA replication is followed by transient strand undermethylation which serves in strand discrimination. MutS recognizes and binds to the mismatch, followed by binding and hydrolysis of ATP. The consequences are two-fold : formation of a MutS-stabilized α -loop and a conformational change which allows MutL to bind and translocate along the DNA towards the nearest hemimethylated d(GATC) site. MutH joins the complex and is activated to nick 5' to the site. Unwinding of the incised strand by DNA helicase II is followed by exonucleolytic degradation by one of three enzymes. Synthesis and ligation by DNA polymerase III and DNA ligase completes the process. Lastly, Dam methylates the d(GATC) site, preventing any further action by MMR.

between MutL and other ATP-binding proteins such as DNA topoisomerase II and Hsp90 resulting in the classification of all members in the newly created GHKL ATPase/kinase superfamily (Dutta & Inouye, 2000).

The MutS-MutL repair complex, combined with the action of ATP hydrolysis, precipitates the activation of MutH (Au *et al.*, 1992). Interestingly, MutL alone, at high concentrations, is capable of activating MutH in an ATP-dependent manner (Ban & Yang, 1998). MutH manifests a latent endonuclease activity necessitating Mg^{2+} as a cofactor (Welsh *et al.*, 1987). This 25 kDa monomer is believed to cleave 5' to the d(GATC) tetranucleotide sequence (Welsh *et al.*, 1987) located closest to the mispair (Au *et al.*, 1992). Requirements for the presence of MutH and the d(GATC) stretch serving as the strand discrimination signal can be bypassed by introducing an incision in the undermethylated strand (Lahue *et al.*, 1989).

Nicking of the undermethylated strand by MutH can take place either 3' or 5' to the mismatch (Au *et al.*, 1992). The choice of which single-stranded exonuclease will carry out the excision and degradation of the DNA between the nick and the mismatch is contingent upon the orientation of said nick (Cooper *et al.*, 1993). An unmethylated d(GATC) site located 5' to the mismatch will require the 5' → 3' exonucleolytic activities of exonuclease VII or RecJ. On the other hand, positioning of the d(GATC) site 3' to the mismatch calls for the action of exonuclease I. However, irrespective of the exonuclease utilized, repair in either orientation is conditional upon the presence of DNA helicase II (Grilley *et al.*, 1993). The product of *uvrD* is responsible for unwinding the DNA at the nick. MutL assists in loading the helicase

onto the DNA as well as aids in the magnification of its activity (Yamaguchi *et al.*, 1998; Dao & Modrich, 1998). Following exonucleolytic degradation, the resulting gap is stabilized by the single-strand binding (SSB) protein. Resynthesis is carried out by DNA polymerase III while DNA ligase covalently reseals the nick. The DNA adenine methylase (Dam) methylates the adenine of the d(GATC) site, thus preventing further action by postreplicative repair.

1.2.3 Mismatch Repair in Eukaryotes

Extensive studies carried out on methyl-directed mismatch repair (MMR) in *Escherichia coli* have lead to the identification of the proteins involved as well as the elucidation of their biochemical functions. These breakthroughs have been instrumental in discovering the existence of MutS and MutL homologues in eukaryotic cells. The mismatch repair enzyme homologues that have been detected in humans, yeast and rodents thus far as well as their counterparts in *Escherichia coli*, are listed in Table 1. Another human MutL homologue, found to bind with hMLH1, was discovered not long ago (Lipkin *et al.*, 2000). MutS and MutL homologues have also been identified in *Xenopus laevis* and *Drosophila melanogaster* (Modrich & Lahue, 1996). Recently, MutS homologues in *Arabidopsis thaliana* have been uncovered (Culligan & Hays, 1997; Adé *et al.*, 1999). Of greater importance is that defects in human mismatch repair have been linked with a predisposition to hereditary nonpolyposis colon cancer (HNPCC) as well as ovarian cancer. Moreover, MMR has been associated with the triggering of programmed cell

Table 1. Mismatch Repair Homologues in Eukaryotes

Bacteria (<i>Escherichia coli</i>)	Yeast (<i>Saccharomyces cerevisiae</i>)	Rodent	Human
MutS	MSH2	-	hMSH2
MutS	-	-	hMSH3
MutS	-	Gtmbp	hMSH6 / GTBP*
MutS	MSH3	Rep-3	Duc-1
MutL	PMS1	-	hPMS1
MutL	PMS1	-	hPMS2
MutL	MLH1	-	hMLH1

* G-T mismatch binding protein

death (PCD). The presence of all of these homologues supports the idea that mismatch repair is evolutionarily conserved. Furthermore, it reflects the notion that a more complex genome would require a wider array of repair proteins to maintain its integrity.

Eukaryotic mismatch repair has been well reviewed (Jiricny, 1998; Kolodner & Marsischky, 1999; Jiricny & Nyström-Lahti, 2000). Briefly, eukaryotes possess heterodimeric protein complexes to aid in the removal of lesions similar (*ie.* base-base mispairs, IDLs) to those arising in *Escherichia coli*. Current models for the first step of mismatch repair, recognition and binding, have been outlined in both yeast and humans (Figure 5). (h)MutS α , a recognition complex composed of (h)MSH2 and (h)MSH6, distinguishes both base mispairs and IDLs. A similar complex, (h)MutS β , comprised of (h)MSH2 and (h)MSH3, also exists but binds preferentially to IDLs. Similarly to *Escherichia coli*, human mismatch repair complexes exhibit ATPase activity. Such activity in hMutS α induces the complex to undergo a conformational change into a sliding clamp, allowing translocation along the DNA. Assembly of the repairosome, the second step in the repair, may be triggered by this conformational change. MutL homologues also exist and form complexes (MutL α and MutL β) comparable to those reconstituted by their fellow MutS homologues. MutL α is proposed to be the primary complex involved in binding to either MutS α , MutS β or PCNA (proliferating cell nuclear antigen). PCNA, which can bind to either MutL or MutS homologues, is reported to be involved in strand discrimination by virtue of its association with DNA polymerase δ . This association with the replication complex

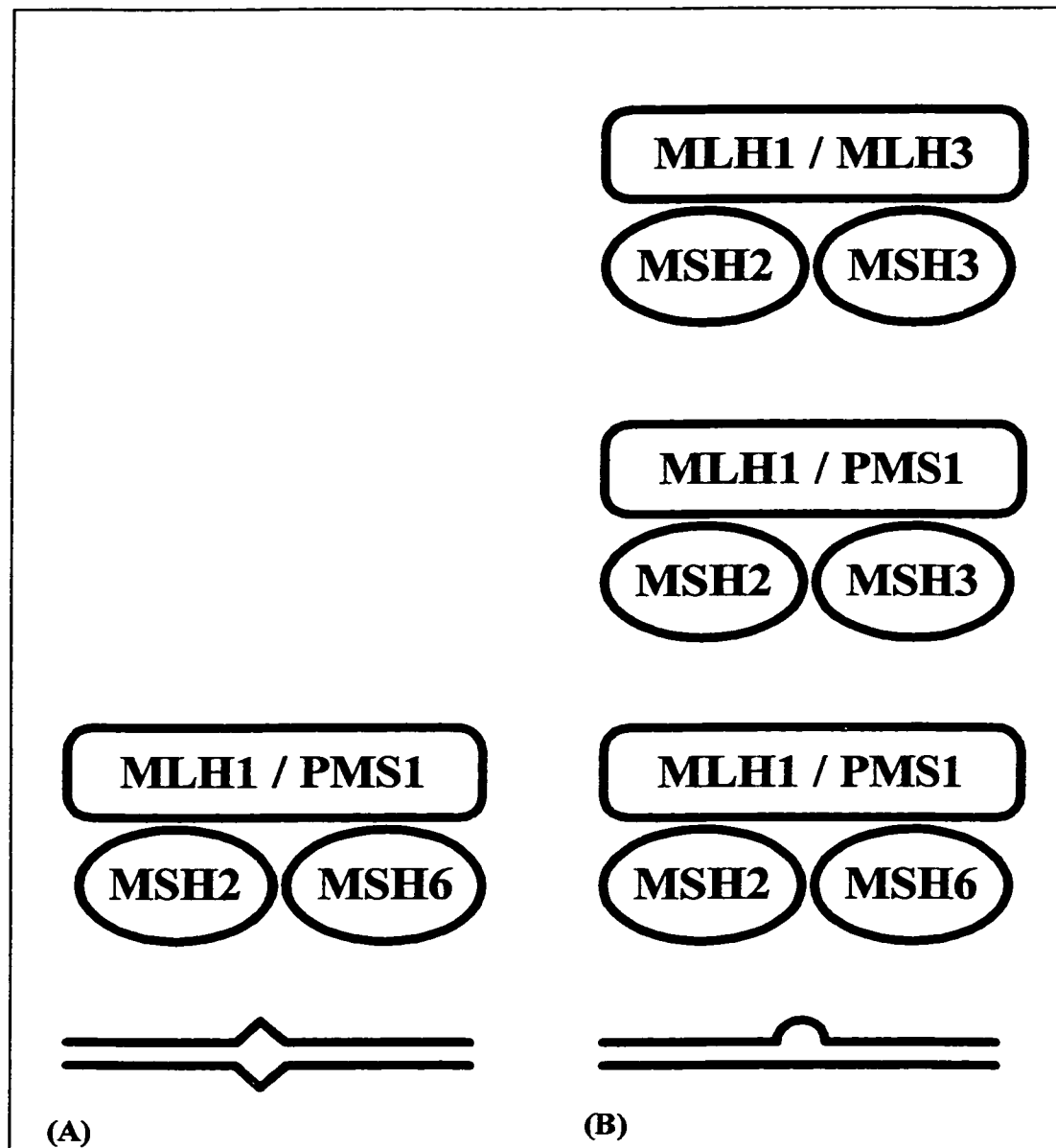


Fig. 5. Specificities of MutS- and MutL-related complexes. Heterotrimeric complexes, composed of different combinations of subunits, are responsible for the repair of either **(A)** Base mispairs or **(B)** Insertion-deletion loops (IDLs).

may aid in recognition of the nascent strand. DNA polymerase δ , replication protein A (RPA) and replication factor C (RFC) as well as the exonuclease EXOI/HEX1 (homologous to ExoI in yeast) have been implicated in this process. No MutH homologues have been reported to date.

1.2.4 Competition Between MMR and VSP For the Repair of a T-G Mismatch

Comparison of the long patch (MMR) and short patch (VSPR) mismatch repair systems discussed above reveals that they differ in many aspects. The length of the DNA tracts removed varies from as few as 10 nucleotides (VSPR) to over one kilobase (MMR). VSPR and MMR stand apart in other ways including the number and types of substrates dealt with and the specific context requirement within which the lesion must be located. VSPR recognizes and repairs only one lesion, a T/G mismatch which, if left uncorrected, will lead to a CG→TA transition mutation. This mismatch will be recognized and repaired only if located within CTWGG or related sequences. MMR recognizes all eight possible mismatches and IDLs of up to four nucleotides. Repair of each is undertaken with the sole exception of the C/C mismatch. Sequence context requirements for repair have not been reported. MMR takes advantage of brief strand undermethylation following synthesis to direct repair to the correct strand. VSPR has no such requirement. It is a methylation-independent base-specific repair system that removes the thymine regardless of what strand it is located on.

Irrespective of this diversity of features, each system retains in common with

the other the ability to repair the same substrate, a T/G mismatch, with great efficiency. Possible competition between these two systems has been reported (Zell & Fritz, 1987). T/G mispairs generally come to pass due to an error in DNA replication (Figure 6). Ordinarily, postreplicative mismatch repair would direct repair using the methylated strand as a template. According to this scenario, if this same T/G mismatch were to occur within the context of a CCWGG sequence, correction by MMR would result in a T·A pairing or a C·G pairing. VSP repair outcompetes MMR at these sites (Zell & Fritz, 1987). As such, the VSP mechanism of repair dominates at these sites and repair of the lesion will result in the restoration of the original C·G pair.

1.3 Protein-Protein Interactions

Proteins function in a myriad of roles. There exist very few biological systems that do not involve protein interactions of some kind. DNA repair and its associated systems are no exception. Traditional biochemical methods for detecting protein interactions include co-immunoprecipitation, chemical crosslinking and affinity chromatography. Drawbacks to the utilization of these methods include the time consuming methodology involved and the fact that the great majority of these protein-protein complexes can only be studied *in vitro*.

Studies involving protein interactions were revolutionized by the advent of two-hybrid systems as pioneered by Fields and Song (1989). Such systems take

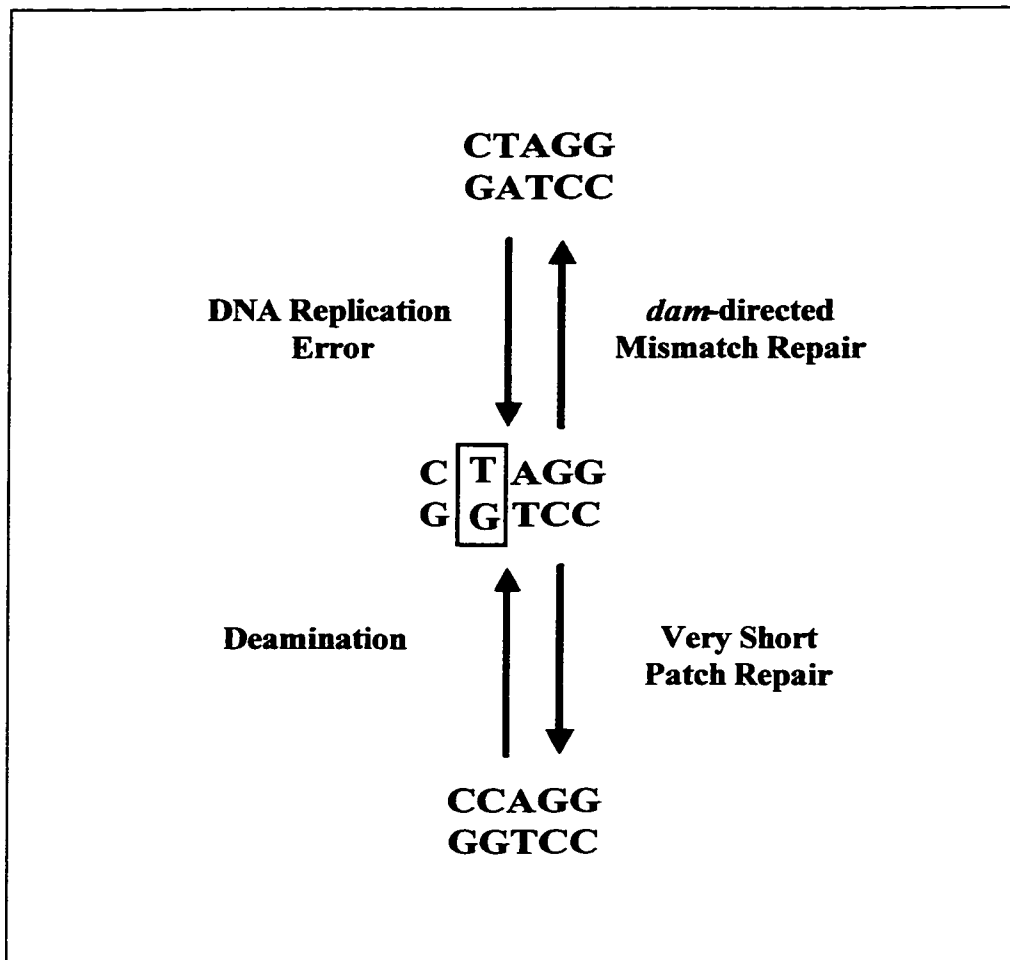


Fig. 6. Competition between *dam*-directed mismatch repair (MMR) and very short patch (VSP) repair. T/G mismatches arise from either DNA replication errors or from the deamination of 5-methylcytosine located within a Dcm recognition site. MMR processes the lesion arising in the former situation while VSP repair is responsible for correction in the latter scenario. However, were MMR to repair the lesion in the context of a recognition site, correction would result in a T·A pairing or a C·G pairing depending on which strand is used as template. Therefore, unwanted mutation is avoided by having VSP repair outcompete MMR for T/G mismatches located within CCWGG sequences.

advantage of the modular nature of many transcriptional activators (Figure 7). These. contain a DNA binding (DB) domain, which binds the promoter, and a transcriptional activation domain (AD), which recruits the transcription machinery in the host, usually yeast. Ordinarily, both domains reside on the same protein. However, they remain functional and retain the ability to initiate transcription as long as they find themselves in close proximity. Proteins to be tested are fused to the two separated domains. If the proteins interact, then a functional transcription machinery is reconstituted. This action drives the expression of a reporter gene such as *lacZ*.

1.3.1 Bacterial Two Hybrid System

The same simple principle on which the yeast two hybrid system is based has been applied to bacteria. Adenylate cyclase (AC, *cyaA*) of *Bordetella pertussis* catalyzes the formation of cyclic AMP (cAMP) from ATP. cAMP in turn binds to the catabolite activating protein (CAP). The CAP/cAMP complex can then recognize specific promoters and switch on the transcription of genes such as *lacZ* which are involved in carbohydrate fermentation. Proper functioning of the AC catalytic domain is dependent upon the presence of calmodulin (Ladant, 1988). Past studies revealed that, after limited proteolysis, the catalytic domain can be divided into two fragments, T18 and T25 (Ladant, 1988). Reconstitution of the fragments, in the presence of calmodulin, leads to a functional protein.

The creation of the bacterial two hybrid system takes advantage of the modular nature of this catalytic domain. (Figure 8) (Karimova *et al.*, 1998).

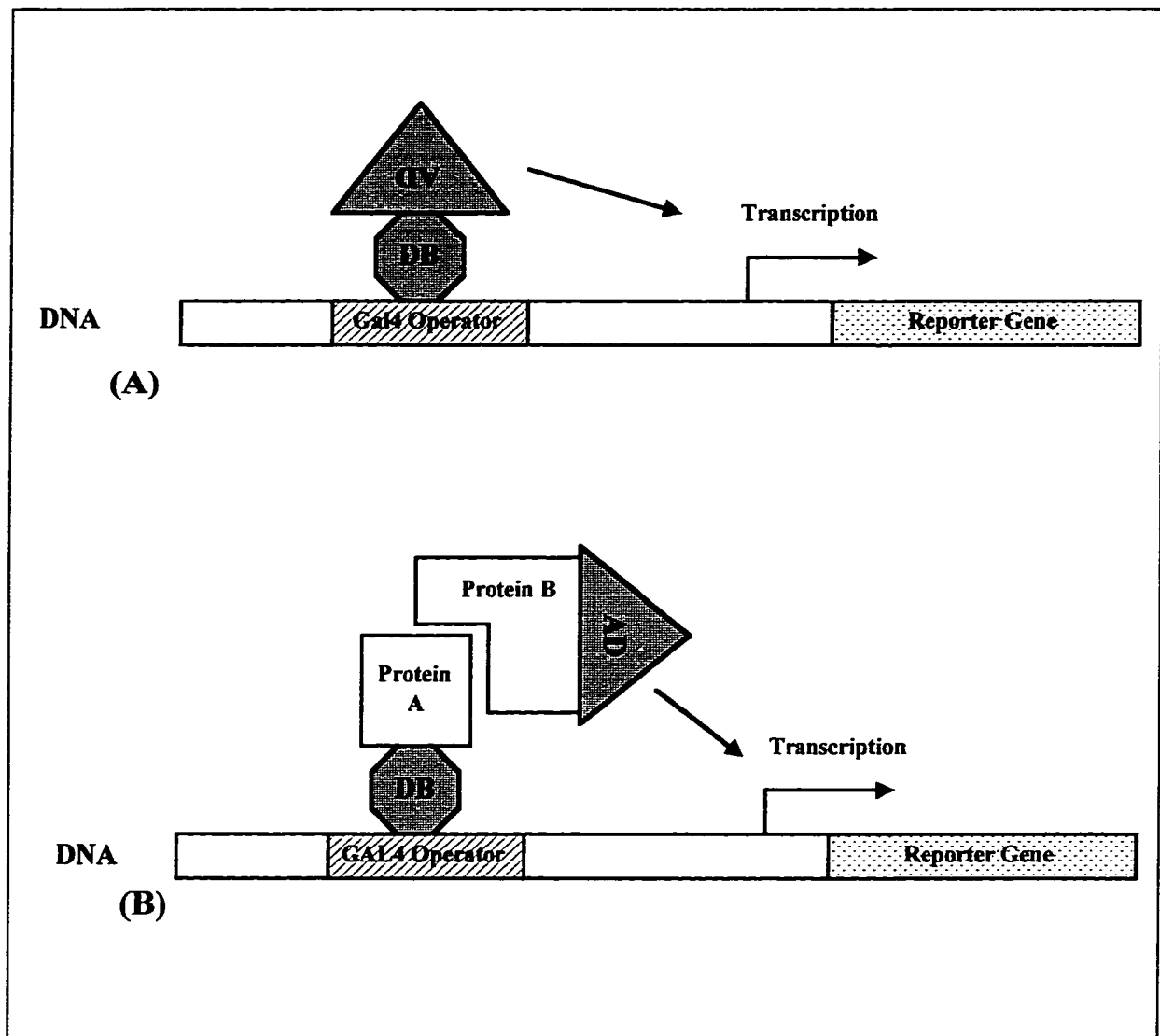


Fig. 7. The yeast two hybrid (Y2H) system. (A) Eukaryotic transcriptional activators are modular in nature. They are characterized by two dissociable functional domains. The DNA binding (DB) domain interacts with DNA and is linked to the activation domain (AD) to cause transcription. (B) The Y2H system uses the yeast Gal4 transcriptional activator which is required for expression of genes encoding proteins involved in galactose metabolism. Interaction of Protein A and Protein B brings the DNA binding and activation domains in close enough proximity to allow transcription of the reporter gene.

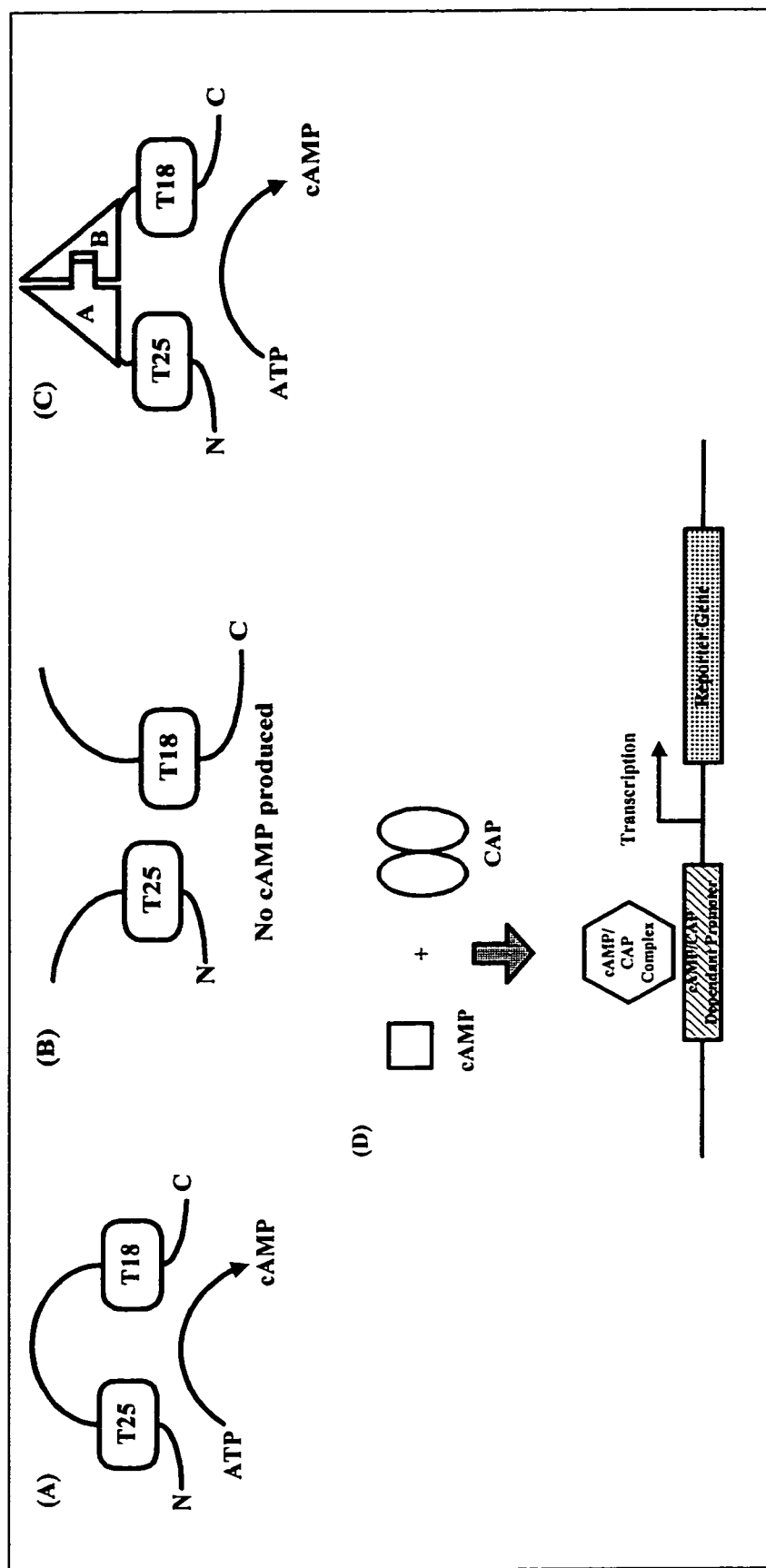


Fig. 8. The bacterial two hybrid (B2H) system. (A) The full-length catalytic domain of *Bordetella pertussis* adenylate cyclase exhibits a constitutive enzymatic activity that results in cAMP synthesis. (B) The two fragments, T25 and T18, when co-expressed as independent polypeptides, are unable to interact and no cAMP synthesis occurs. (C) When the two fragments are fused to interacting proteins A and B, they are brought in close proximity thus reconstituting catalytic activity followed by cAMP production. (D) cAMP, synthesized in an *E. coli cyaA* strain by the complementing T25 and T18 pairs, binds to the catabolite activator protein (CAP). The cAMP/CAP complex can then recognize specific promoters and switch on the transcription of the *mal* genes or *lacZ*.

Independent expression of T18 and T25 in an *Escherichia coli cyaA* strain results in their inability to recognize each other and no cAMP synthesis occurs. If each of the fragments are fused to putatively interacting proteins, the former are brought in close enough proximity so as to allow the reconstitution of catalytic activity. Interactions can be selected for on plates containing Xgal. This assay is of great interest because it allows for *in vivo* experimentation. It does not require purified proteins or prior knowledge of the protein's function. In addition, it allows for easy retrieval of the genes of interest since they are contained in the clones that scored positively.

1.3.2 Known Interactions Between MMR and VSP Repair Proteins

Despite all the players acting in postreplicative mismatch repair having been revealed and the system's *modus operandi* by and large described, little is known about the specific individual steps which define this critical process. Studying the influence of protein-protein complexes formed between system members at each point can aid in clarifying these.

Sedimentation experiments revealed that MutS (Su *et al.*, 1986), and MutL (Grilley *et al.*, 1989), regarded as a possible molecular matchmaker (Sancar & Hearst, 1993), exist in a homodimeric form. Mut S also possesses the ability to oligomerize (Su *et al.*, 1986; Allen *et al.*, 1997, Bjornson *et al.*, 2000). DnaseI footprinting analysis (Grilley *et al.*, 1989) and electron microscopy studies (Allen *et al.*, 1997) uncovered the interaction between MutL and MutS. Earlier attempts to detect a MutS-MutL protein complex using co-immunoprecipitation proved to be unsuccessful (Wu &

Marinus, unpublished as cited in Wu & Marinus, 1999). Likewise, this complex as well as the dimerization of MutS and MutL was not observed using the yeast two hybrid system (Hall & Matson, 1999).

Deletion analysis of MutS established that its C-terminus is responsible for dimerization as well as its interaction with MutL while its N-terminus is responsible for DNA binding (Wu & Marinus, 1999). In addition to complexing with MutS, MutL binds with MutH (Hall & Matson, 1999) and DNA helicase II (Hall *et al.*, 1998). Amino acids in the C-terminal region of MutL are responsible for dimerization (Drotschmann *et al.*, 1998) as well as interaction with DNA helicase II (Hall *et al.*, 1998). Interaction between MutS and MutH has not been reported although interaction between MutL and MutH has been observed using the yeast two hybrid system (Hall & Matson, 1999). Crystallization of MutH lead to the proposition that its C-terminal tail may constitute the location of interaction between itself and MutL/MutS (Ban & Yang, 1998)

A certain amount of overlap exists between mismatch repair systems. Very short patch repair (VSPR) activity is severely impaired in strains that are *mutS* or *mutL* (Lieb, 1987). Drotschmann *et al.* (1998) presented evidence that MutL greatly enhances not only Vsr activity but MutS activity also. This suggested that there may be contact between MutL and Vsr. Interactions between these were first seen in the yeast two hybrid system (Doiron & Cupples, unpublished).

2.0 Materials and Methods

2.1 Bacterial Strains and Media

All bacterial strains and plasmids used are listed in Tables 2 and 3, respectively. Unless otherwise noted, cells from a single colony of each strain were grown overnight at 37°C with aeration. Rich medium was made with Bacto LB broth, Miller (Difco) at 25 g per litre. Minimal media was assembled as described in Miller (1992). When necessary, 16 g of agar (Sigma) per litre was supplied for media solidification. Antibiotics or supplements were added as required according to the concentrations listed in Table 4.

2.2 Strain Construction

Isogenic strains LJ2809 *mutS*::Tn5, LJ2809 *mutL*::Tn5, and LJ2809 *mutH*::Tn5 were constructed by generalized transduction using P1vir, as adapted from the protocol outlined in Miller (1992). The *mutH*::Tn5, *mutL*::Tn5 and *mutS*::Tn5 lysates were obtained from K. Doiron. An equivalent volume of MC buffer (0.1 M MgSO₄, 0.005 M CaCl₂) was added to a 5 ml overnight of LJ2809 and aerated at 37°C for 45 minutes. Six microfuge tubes were set up as follows and incubated at 37°C for 30 minutes.

- Tube 1: 100 µl cells
- Tube 2 : 100 µl cells + 100 µl lysate
- Tube 3 : 100 µl cells + 100 µl lysate (10⁻¹ dilution)
- Tube 4 : 100 µl cells + 100 µl lysate (10⁻² dilution)
- Tube 5 : 100 µl cells + 100 µl lysate (10⁻³ dilution)
- Tube 6 : 100 µl lysate

Table 2. Strains Used

Strain Designation	Genotype	Reference
P90C	<i>ara, Δ(gpt-lac)5, thi</i>	Cupples & Miller, 1988
LJ2809	<i>fruR11::Tn10, xylA7, ΔcyaA854, ΔargH1</i>	Glaser <i>et al.</i> , 1989
LJ2809 <i>mutS::Tn5</i>	<i>fruR11::Tn10, xylA7, ΔcyaA854, ΔargH1, mutS::Tn5</i>	This study
LJ2809 <i>mutL::Tn5</i>	<i>fruR11::Tn10, xylA7, ΔcyaA854, ΔargH1, mutL::Tn5</i>	This study
LJ2809 <i>mutH::Tn5</i>	<i>fruR11::Tn10, xylA7, ΔcyaA854, ΔargH1, mutH::Tn5</i>	This study
∞ S90C <i>mutS::Tn5</i>	<i>ara, Δ(gpt-lac)5, thi, rpsL, mutS::Tn5</i>	Macintyre <i>et al.</i> , 1997
S90C <i>mutL::Tn5</i>	<i>ara, Δ(gpt-lac)5, thi, rpsL, mutL::Tn5</i>	Macintyre <i>et al.</i> , 1997
S90C <i>mutH::Tn5</i>	<i>ara, Δ(gpt-lac)5, thi, rpsL, mutH::Tn5</i>	Macintyre <i>et al.</i> , 1997
P90C-V	<i>ara, Δ(gpt-lac)5, thi, vsr::kan F' lacZ proA⁺B⁺ (pro462)</i>	This lab, unpublished
CC112-V	<i>ara, Δ(gpt-lac)5, thi, gyrA, argE(Am), rpoB, vsr::kan F' lacZ proA⁺B⁺, pGFIB (CCAGG)</i>	Doiron <i>et al.</i> , 1999

Table 3. Plasmids Used

Name	Essential Features	Reference
pT18	Derivative of pBluescript™ II KS, Amp ^r , contains CyaA T18 fragment (aa 225-399) under the control of the <i>lacUV5</i> promoter	Karimova <i>et al.</i> , 1998
pT25	Derivative of pACYC184, Cm ^r , contains CyaA T25 fragment (aa 1-224) under the control of the <i>lacUV5</i> promoter	Karimova <i>et al.</i> , 1998
pT18zip	Derivative of pT18, contains 35 aa leucine zipper fused to N-terminus of CyaA T18 fragment (aa 225-399)	Karimova <i>et al.</i> , 1998
pT25zip	Derivative of pT25, contains 35aa leucine zipper fused to C-terminus of CyaA T25 fragment (aa 225-399)	Karimova <i>et al.</i> , 1998
pTS18	Derivative of pT18, contains <i>mutS</i> fused to N-terminus of CyaA T18 fragment	This study
pTS25	Derivative of pT25, contains <i>mutS</i> fused to C-terminus of CyaA T25 fragment	This study
pTL18	Derivative of pT18, contains <i>mutL</i> fused to N-terminus of CyaA T18 fragment	This study
pTL25	Derivative of pT25, contains <i>mutL</i> fused to C-terminus of CyaA T25 fragment	This study
pTH18	Derivative of pT18, contains <i>mutH</i> fused to N-terminus of CyaA T18 fragment	This study
pTH25	Derivative of pT25, contains <i>mutH</i> fused to C-terminus of CyaA T25 fragment	This stud
pTV18	Derivative of pT18, contains <i>vsr</i> fused to N-terminus of CyaA T18 fragment	This study
pTV25	Derivative of pT25, contains <i>vsr</i> fused to C-terminus of CyaA T25 fragment	This study
pTSV18	Derivative of pTS18, Amp ^r , fragment from pKK-V containing <i>vsr</i> under the control of the <i>trc</i> promoter subcloned into <i>BamHI</i> site	This study

Table 3 (continued). Plasmids Used

Name	Essential Features	Reference
pTLV18	Derivative of pTL18, Amp ^r , fragment from pKK-V containing <i>vsr</i> under the control of the <i>trc</i> promoter cloned into <i>Bam</i> HI site	This study
pTHV18	Derivative of pTH18, Amp ^r , fragment from pKK-V containing <i>vsr</i> under the control of the <i>trc</i> promoter cloned into <i>Bam</i> HI site	This study
pDVW	Derivative of pBR322, Amp ^r , contains fragment from CC112 incorporating <i>dcm</i> and <i>vsr</i>	Petropoulos <i>et al.</i> , 1994
pBR322	Amp ^r	Sambrook <i>et al.</i> , 1989
pDV108	Derivative of pACYC184, Amp ^r , contains <i>vsr</i> under the control of the <i>trc</i> promoter	Macintyre <i>et al.</i> , 1997
pACYC184	Tet ^r , Cm ^r	Sambrook <i>et al.</i> , 1989
pKK-V	Derivative of pKK233-2, Amp ^r , contains <i>vsr</i> under the control of the <i>trc</i> promoter	Doiron <i>et al.</i> , 1996
pKK233-2	Amp ^r	Sambrook <i>et al.</i> , 1989
pETV	Derivative of pET15b (Novagen), Amp ^r , contains a hexahistidine tag fused to the N-terminus of <i>vsr</i>	Cupples & Macintyre, 1999
pMQ348	Derivative of pACYC184, Cm ^r , <i>mutH</i>	Wu & Marinus, 1994
pMQ339	Derivative of pACYC184, Cm ^r , <i>mutL</i>	Wu & Marinus, 1994
pMQ341	Derivative of pACYC184, Cm ^r , <i>mutS</i>	Wu & Marinus, 1994

Table 4. Antibiotics and Supplements Used

Antibiotics and Supplements	Final Concentration
Ampicillin (Fisher Scientific)	100 µg/ml
Chloramphenicol (Boehringer Mannheim)	30 µg/ml
Kanamycin (Fisher Biotech)	50 µg/ml
Rifampicin (ICN Biomedicals Inc.)	100 µg/ml
Tetracycline (Boehringer Mannheim)	15 µg/ml
IPTG (isopropyl-β-D-thiogalactopyranoside, Boehringer Mannheim)	0.5 mM
Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Bachem Inc.)	40 µg/ml
ONPG (<i>o</i> -Nitrophenyl-β-D-galactosidase, Boehringer Mannheim)	4 mg/ml

After the requisite time had elapsed, phage readsorption was prevented by the addition of 200 μ l 1 M sodium citrate (pH 5.5). The Eppendorf tubes were then centrifuged (Canlab Biofuge A) at 13000 rpm for 90 seconds. After the removal of the superjacent, the pellet was resuspended in 100 μ l of LB medium and spread on appropriate selective plates. Resulting transductants were single colony purified. The presence of the mutator alleles was monitored by increased frequency of rifampicin resistance at 37°C.

2.3 Competent Cells

Cells were made competent via the calcium chloride method (Sambrook *et al.*, 1989). A 250 ml Erlenmeyer flask containing 25 ml of LB medium supplemented with appropriate antibiotics was inoculated with 0.25 ml of an overnight culture. The newly inoculated culture was placed in a shaker incubator (New Brunswick Scientific Co.) until the OD₆₀₀ had reached a value between 0.2 and 0.25. The cells were first placed on ice for 10 minutes so as to stop cell growth. Centrifugation of the culture at room temperature for 8 minutes at 3000 rpm (International Equipment Company HN-SII clinical centrifuge) followed. The supernatant was removed and the pellet resuspended in 12.5 ml ($\frac{1}{2}$ of the original volume) of cold 50 mM CaCl₂. The cell suspension was cooled on ice for 30 minutes and then re-centrifuged under the same conditions stated above. Removal of the supernatant preceded resuspension of the cell pellet in 2.5 ml ($\frac{1}{10}$ of the original volume) of cold 50 mM CaCl₂.

2.4 Transformation of Competent Cells with Plasmid DNA

Introduction of plasmid DNA into competent cells was carried out using the protocol indicated in Sambrook *et al.* (1989). Competent cells (200 µl) and plasmid DNA (2 µl) were gently mixed together in a microfuge tube and incubated on ice for 45 minutes. Once the required time had elapsed, the mixture was placed in a waterbath set at 42°C and heatshocked for 90 seconds. The tube was then placed on ice and 0.8 ml LB added. Incubation in a 37°C waterbath for 1 hour followed so as to allow antibiotic expression. The mixture was centrifuged at room temperature for 3 minutes at 13000 rpm. The resulting pellet was resuspended in 0.1 ml of LB, spread on appropriate selective plates and incubated at 37°C for 16 to 48 hours. Four transformants were selected and single colony purified for future use.

2.5 Extraction of Plasmid DNA from Cells

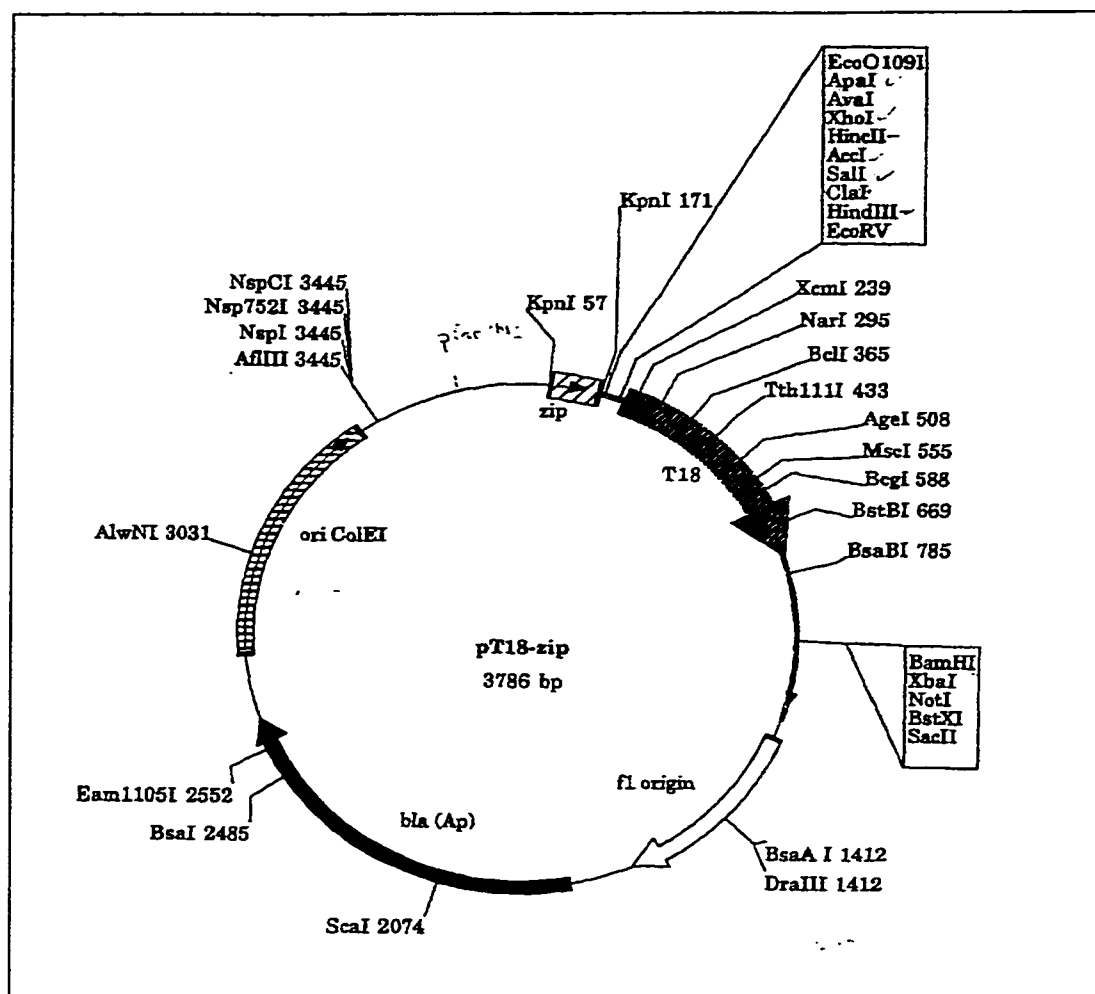
The alkaline lysis method (Sambrook *et al.*, 1989) was the protocol of choice for the small-scale preparation of plasmid DNA. All steps were carried out at room temperature. An overnight culture was transferred to a microfuge tube and centrifuged for 4 minutes at 13000 rpm. The supernatant was removed, the pellet resuspended in 0.1 ml of Solution I (50 mM glucose, 25 mM Tris·Cl [pH 8.0], 10 mM EDTA [pH 8.0]) and incubated for 5 minutes. Once the time requirement was fulfilled, 0.2 ml of freshly made Solution II (0.2 N NaOH; 1% SDS) was added. Following vigorous vortexing, the mixture was left to incubate for another 5 minutes. Finally, 0.15 ml of Solution III was added, the contents mixed by rapid inversion and the suspension

incubated for another 5 minute stretch. The sample was centrifuged (Canlab Biofuge A) for 4 minutes at 13000 rpm. The supernatant was removed and added to another microfuge tube containing 0.4 ml phenol. The components were well vortexed and re-centrifuged for 4 minutes at 13000 rpm so as to separate the aqueous and organic layers. The top (aqueous) layer was removed and placed in another microfuge tube containing 0.8 ml cold 95% ethanol. To the remaining layer, 0.2 ml of ddH₂O was added. The two were well vortexed and centrifuged under the same conditions outlined in the previous step. The microfuge tube was discarded once the aqueous layer was removed and added to the layer previously extracted. The tube was gently inverted a few times and centrifuged using the same parameters as before. The ethanol was removed and the remaining pellet was washed with 70% ethanol and then removed. The DNA pellet was air dried, resuspended in 40 µl ddH₂O and frozen until further use.

2.6 Construction of the Translational Gene Fusion Vectors

2.6.1 Base Vectors (pT25 and pT18)

The vectors pT25zip (Figure 9) and pT18zip (Figure 10) contain amino acids 1-224 (T25) and 225-399 (T18), respectively, of the *B. pertussis* adenylate cyclase catalytic domain (Karimova *et al.*, 1998). The T25 fragment is fused in frame with the N-terminus of a 35 aa leucine zipper. The same leucine zipper is fused in frame with the N-terminus of T18. Removal of this short amino acid stretch by restriction digest using KpnI (MBI Fermentas), enabled the creation of base vectors essential



pT18zip Multicloning Site

Val Pro Gly Pro Pro Ser Arg Ser Thr Val Ser Ile Ser
g gtA CCG GGC CCC CCC TCG AGG TCG ACG GTA TCG ATA AGC
 KpnI || ApaI XhoI || SalI ClaI || HindIII

 Leu Ile Ser
TTG ATA TCG
 || EcoRV

Fig. 9. Plasmid Map of pT18zip. Bacterial two hybrid system vector containing a 35 amino acid leucine zipper fused in frame with the T18 fragment (amino acids 225-399) of the *B. pertussis* adenylate cyclase catalytic domain. The nucleotides in small letters represent the end of the leucine zipper sequence.

for assembling the next set of fusion plasmids.

2.6.2 *mutS* (pTS25 and pTS18), *mutL* (pTL25 and pTL18), *mutH* (pTH25 and pTH18), *vsr* (pTV25 and pTV18)

Amplification of the *mutS* coding region was achieved using the Expand™ Long Template PCR System (Boehringer Mannheim) with pMQ341 as its target template. Oligonucleotide primers (BioCorp., Table 5) contained *Bam*HI and *Sma*I restriction enzyme sites that allowed the insertion of *mutS* into the multicloning site (MCS) of pT25. The elements which composed the reaction as well as the PCR program settings utilized (Bellco Biotechnology DNA Pacer) are described in Table 6. Each polymerase chain reaction (PCR) was carried out in duplicate. A 0.7% 1X TAE agarose verification gel confirmed that the correct amplified PCR products were obtained. Recovery and purification of the PCR product (insert) was carried out using the GeneClean® II extraction kit (Bio101 Inc.).

Both vector and insert were first digested with *Bam*HI (MBI Fermentas). Again, a 0.7% 1X TAE agarose gel revealed that proper digestion had ensued. DNA purification of both insert and vector was ensured by the use of the Wizard™ DNA Clean-Up System (Promega). *mutS* and pT25 were then subjected to digestion by *Sma*I (MBI Fermentas) and purified in the same manner as before. Shrimp alkaline phosphatase (Amersham Life Sciences) was used to remove the terminal 5' phosphate groups from the ends of the vector. Insert and dephosphorylated vector, combined in

Table 5. Oligonucleotides Used

Primer #	Sequence	Function
177	5' CGGACAGGAT <u>CCCATGAGTG</u> 3'	To insert a Bam HI site in frame at the 5' end of <i>mutS</i> for cloning into pT25
178	5' AAGACTA <u>CCCCGGGATTGTTA</u> 3'	To insert a Sma I site in frame at the 3' end of <i>mutS</i> for cloning into pT25
179	5' AATATCAGGG <u>CCCCCGGACAT</u> 3'	To insert an Apa I site in frame at the 5' end of <i>mutS</i> for cloning into pT18
186	5' GAATTGTG <u>TCCGACAC</u> CAGGCTC 3'	To insert a Sal I site in frame at the 3' end of <i>mutS</i> for cloning into pT18
181	5' AACTAAG <u>CCCCGGGTGATGCC</u> 3'	To insert a Sma I site in frame at the 5' end of <i>mutL</i> for cloning into pT25
182	5' TTAGGCAGGTA <u>CCCCCTTACT</u> 3'	To insert a Kpn I site in frame at the 3' end of <i>mutL</i> for cloning into pT25
183	5' CAAACTAGGG <u>CCCCATTGATG</u> 3'	To insert an Apa I site in frame at the 5' end of <i>mutL</i> for cloning into pT18
184	5' TTA <u>CTGAAAGCTTTCATCTTT</u> 3'	To insert a Hind III site in frame at the 3' end of <i>mutL</i> for cloning into pT18

Table 5 (continued). Oligonucleotides Used

Primer #	Sequence	Function
191	5' GCAAGAGGGAT <u>CCCATGGCC</u> 3'	To insert a Bam HI site in frame at the 5' end of <i>vsr</i> for cloning into pT25
192	5' CAGTTAT <u>CCCGGGCGCATCA</u> 3'	To insert a Sma I site in frame at the 3' end of <i>vsr</i> for cloning into pT25
193	5' TCAGCAAGGGCC <u>CCCAACATG</u> 3'	To insert an Apa I site in frame at the 5' end of <i>vsr</i> for cloning into pT18
194	5' GGACGAAGCTT <u>GCGAGTAAAT</u> 3'	To insert a Hind III site at the 3' end of <i>vsr</i> for cloning into pT18
195	5' TCAAGGTCCCGG <u>GACATGTCC</u> 3'	To insert a Sma I site in frame at the 5' end of <i>mutH</i> for cloning into pT25
196	5' AGCGGCAGGTAC <u>CCAGCGATG</u> 3'	To insert a Kpn I site in frame at the 3' end of <i>mutH</i> for cloning into pT25
197	5' ATCAAGGGGGCC <u>CTGACATGT</u> 3'	To insert an Apa I site in frame at the 5' end of <i>mutH</i> for cloning into pT18
198	5' AAGCGATGTCGAC <u>TGGATCA</u> 3'	To insert a Sal I site in frame at the 3' end of <i>mutH</i> for cloning into pT18

Table 6. PCR Reaction Elements and Program Parameters

Goal	PCR Reaction Mixtures	PCR Program Parameters
Cloning <i>mutS</i> into pT18	<ul style="list-style-type: none"> • 0.5 µl Plasmid DNA (pMQ341) • 5.0 µl 3.5 mM dNTPs • 1.0 µl Oligo #179 • 1.0 µl Oligo #186 • 5.0 µl 10X PCR Buffer 1 (17.5 mM MgCl₂) • 37.0 µl ddH₂O • 0.5 µl Taq Expand Polymerase <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 30 seconds • <u>Annealing</u> : 50°C for 2 minutes • <u>Extension</u> : 68°C for 3 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>
Cloning <i>mutS</i> into pT25	<ul style="list-style-type: none"> • 0.5 µl Plasmid DNA (pMQ341) • 5.0 µl 3.5 mM dNTPs • 1.0 µl Oligo #177 • 1.0 µl Oligo #178 • 5.0 µl 10X PCR Buffer 1 (17.5 mM MgCl₂) • 37.0 µl ddH₂O • 0.5 µl Taq Expand Polymerase <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 30 seconds • <u>Annealing</u> : 47°C for 2 minutes • <u>Extension</u> : 68°C for 3 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>

Table 6 (Continued). PCR Reaction Elements and Program Parameters

Goal	PCR Reaction Mixtures	PCR Program Parameters
Cloning <i>mutL</i> into pT18	<ul style="list-style-type: none"> • 4.0 µl Plasmid DNA (pMQ338) • 5.0 µl 3.5 mM dNTPs • 1.0 µl Oligo #183 • 1.0 µl Oligo #184 • 5.0 µl 10X PCR Buffer (20 mM MgCl₂) • 33.5.0 µl ddH₂O • 0.5 µl Tax Polymerase (MBI Fermentas) <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 1 minute • <u>Annealing</u> : 43°C for 2 minutes • <u>Extension</u> : 72°C for 2 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>
Cloning <i>mutL</i> into pT25	<ul style="list-style-type: none"> • 4.0 µl Plasmid DNA (pMQ338) • 5.0 µl 2.0 mM dNTPs • 1.0 µl Oligo #181 • 1.0 µl Oligo #182 • 5.0 µl 10X PCR Buffer (15 mM MgCl₂) • 33.5 µl ddH₂O • 0.5 µl Tax Polymerase (MBI Fermentas) <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 1 minute • <u>Annealing</u> : 60°C for 2 minutes • <u>Extension</u> : 72°C for 2 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>

Table 6 (Continued). PCR Reaction Elements and Program Parameters

Goal	PCR Reaction Mixtures	PCR Program Parameters
Cloning <i>mutH</i> into pT18	<ul style="list-style-type: none"> • 4.0 µl Plasmid DNA (pMQ348) • 5.0 µl 2.0 mM dNTPs • 1.0 µl Oligo #197 • 1.0 µl Oligo #198 • 5.0 µl 10X PCR Buffer (15 mM MgCl₂) • 37.0 µl ddH₂O • 0.5 µl Taq Polymerase (MBI Fermentas) <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 30 seconds • <u>Annealing</u> : 47°C for 2 minutes • <u>Extension</u> : 68°C for 2 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>
Cloning <i>mutH</i> into pT25	<ul style="list-style-type: none"> • 4.0 µl Plasmid DNA (pMQ348) • 5.0 µl 2.0 mM dNTPs • 1.0 µl Oligo #195 • 1.0 µl Oligo #196 • 5.0 µl 10X PCR Buffer (15 mM MgCl₂) • 37.0 µl ddH₂O • 0.5 µl Taq Polymerase (MBI Fermentas) <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 30 seconds • <u>Annealing</u> : 53°C for 2 minutes • <u>Extension</u> : 68°C for 2 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>

Table 6 (Continued). PCR Reaction Elements and Program Parameters

Goal	PCR Reaction Mixtures	PCR Program Parameters
Cloning <i>vsr</i> into pT18	<ul style="list-style-type: none"> • 20 µl Chromosomal DNA • 5.0 µl 2.0 mM dNTPs • 1.0 µl Oligo #193 • 1.0 µl Oligo #194 • 5.0 µl 10X PCR Buffer (15 mM MgCl₂) • 17.5 µl ddH₂O • 0.5 µl Taq Polymerase (MBI Fermentas) <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 30 seconds • <u>Annealing</u> : 53°C for 2 minutes • <u>Extension</u> : 68°C for 2 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>
Cloning <i>vsr</i> into pT25	<ul style="list-style-type: none"> • 20 µl Chromosomal DNA • 5.0 µl 2.0 mM dNTPs • 1.0 µl Oligo #191 • 1.0 µl Oligo #192 • 5.0 µl 10X PCR Buffer (15 mM MgCl₂) • 17.5 µl ddH₂O • 0.5 µl Taq Polymerase (MBI Fermentas) <p>* Total reaction volume : 50 µl</p>	<ul style="list-style-type: none"> • <u>Initial denaturation step</u> : 96°C for 4 minutes; 1 cycle • <u>Hold</u> : 72°C for the addition of Tax to each reaction • <u>Denaturation</u> : 95°C for 30 seconds • <u>Annealing</u> : 53°C for 2 minutes • <u>Extension</u> : 68°C for 2 minutes • <u>Final extension</u> : 72°C for 5 minutes; 1 cycle <p>* Denaturation, extension and annealing steps are carried out for 25 cycles</p>

a 3:1 ratio, were subsequently ligated at 14 °C overnight in a reaction containing 0.5 µl T4 DNA ligase (MBI Fermentas). The ligation mixtures were transformed into competent P90C. Transformants were single colony purified. Plasmids recovered from putative clones were screened by digestion with appropriate restriction enzymes. Construction of pTS18 was carried out essentially as specified above with the exception of the vector (pT18) , PCR primers (#179 & #186) and restriction enzymes (*Apa*I [Promega], *Sal*I [MBI Fermentas]) employed.

The creation of the remaining fusion vectors was carried out essentially as delineated for the pTS25 with the exception of primers and restriction enzymes used. Amplification of the *mutL*, *mutH* and *vsr* coding regions was achieved using the PCR reaction elements and program parameters outlined in Table 6. However, the template used for the amplification of *vsr* was P90C chromosomal DNA. It was prepared by incubating a large single colony of P90C in 100 µl of ddH₂O for 20 minutes at 37°C. Further, due to the particular design of the PCR primers Nos. 195 and 196, digestion by *Kpn*I was the essential and required first step in the preparation of pTH25. Reversing the order of enzymes used (ie. digesting with *Sma*I then *Kpn*I) results in the destruction of the *Kpn*I site.

2.6.3 *vsr* Interference Studies (pTSV18, pTLV18 and pTHV18)

pTSV18 was created by subcloning the *Bam*HI fragment encompassing *vsr* under the control of the *trc* promoter originating from pKK-V into pTS18. Likewise, this same fragment was subcloned into pTL18 and pTH18, giving rise to the vectors

pTLV18 and pTHV18, respectively. Standard restriction enzyme and ligation reactions as well as isolation procedures for the putative clones were carried out as stated earlier.

2.7 Determining B2H Fusion Protein Function Using Complementation

Cells deficient in methyl-directed mismatch repair (MMR) are characterized by a distinct mutation spectrum. Previous studies have shown such cells to display increased levels of transitions and frameshifts (Schaaper & Dunn, 1987). Missense mutations caused by malfunctional postreplicative repair can arise in *rpoB*, which codes for the β subunit of RNA polymerase. Alterations in this subunit confer rifampicin resistance (Jin & Gross, 1988). MMR deficiencies can be overcome by introducing a plasmid carrying the intact gene of interest. The presence of these functional repair proteins aids in the prevention of genome modifications such that cells remain rifampicin sensitive.

The goal was to establish whether any of the *mut* gene products existing as part of a fusion protein still retained their ability to complement the mutator phenotype. This phenotype was caused by the presence of a transposon (Tn5) in the same chromosomal *mut* gene as the one present in the fusion. Complementation is reflected in the frequency of spontaneous rifampicin resistant mutants observed.

Each fusion vector, introduced via CaCl_2 -mediated transformation, was tested for this ability. Overnight cultures (100 μl) were spread on rifampicin-containing plates. To determine viability, an identical volume of the same overnight culture, diluted to 10^{-6} , was spread on an LB plate. All plates were incubated overnight at

37°C. Three independent colonies of each strain were assayed in triplicate.

2.8 Lac Reversion : A Complementation Assay for Vsr

Complementation ability of the fusion proteins involving *vsr* was tested in a different manner. Different methodology was necessary because mutations at the Dcm methylation sites in *rpoB* do not result in rifampicin resistance. Glutamic acid, a residue located at position 461 in β -galactosidase, product of *lacZ*, has been reported to be essential for proper enzymatic function (Cupples & Miller, 1988). Based on this, an assay system was designed to detect strains that are deficient in Very Short Patch (VSP) repair. This is achieved by monitoring C-G to T-A transition mutations within specific sequence contexts. In this case, 5-methylcytosines located within the sequence CCAGG were being examined (Ruiz *et al.*, 1993). Site-directed mutagenesis was used to introduce a Dcm methylation recognition sequence centred on codon 461 within the wildtype *lacZ* sequence carried on an episome. Thus, the sequence was changed from AAT GAG TCA (Asn Glu Ser) to AAC CAG GGG (Asn Gln Gly). The resulting Lac⁻ phenotype was attributed to the replacement of glutamic acid by glutamine at the critical codon, 461. When the 5-methylcytosine in this site is deaminated, a TAG stop codon results. If very short patch repair is functional, then the thymine will be recognized, removed and replaced by a cytosine, thus leading to the restoration of the original sequence and the retention of the Lac⁻ phenotype. The TAG mutant would normally also be Lac⁻. However, the effect of the stop codon can be bypassed by introducing the suppressor plasmid pGFIB which

carries a synthetic tRNA gene. The glutamic acid suppressor recognizes the amber stop codon and inserts a glutamic acid, resulting in a Lac⁺ phenotype.

An unfortunate drawback to the assay just described is that transforming with two plasmids is complicated by the already present suppressor plasmid. Moreover, any plasmid that is introduced must have an origin of replication compatible with that of pGFIB. These situations can be circumvented by using a modified version of the assay (Petropoulos *et al.*, 1994). The serine (TCA) at codon 462 of the wildtype *lacZ* sequence carried on an episome was replaced by a proline (CCA), resulting in a Lac⁻ phenotype. A CCAGG sequence is generated since codon 463 is a glycine (GGC). In summary, the sequence at codons 461 to 463 becomes GAG CCA GGC (Glu Pro Gly). Deamination of the 5-methylcytosine located at the second C in the Dcm recognition site will result in the appearance of a leucine codon (CTA) and the production of functional β -galactosidase. Strains that are *vsr*⁺, and hence proficient in VSP repair, will have a low rate of Lac reversion since the thymine is removed and replaced by a cytosine, thus restoring the CCAGG sequence and its associated phenotype. On the other hand, the thymine is not removed in cells without a functional endonuclease. The end result will be the production C·G to T·A transition mutation following the next round of replication. One can expect a high Lac reversion rate in such cells.

Overnight cultures (100 μ l) of either CC112-V containing the fusion vector pTV25 or P90C-V containing either pTV25 or pTV18 were spread on minimal lactose plates. Viability was determined by spreading an identical volume of the same

overnight cultures, diluted to 10^{-6} , on LB plates. All plates were incubated at 37°C. LB plates were left overnight while the incubation time for minimal lactose plates ranged between 24 to 36 hours. Three independent colonies of each strain were assayed in triplicate.

2.9 Screening for *in Vivo* Protein-Protein Interactions - Qualitative Indications

Potential interactions between the components of MMR themselves as well as their interactions with Vsr were screened in LJ2809 and its isogenic strains. All possible pairwise combinations were cotransformed into the individual competent strains. Four transformants were selected and single colony purified on appropriate selective plates. Overnight cultures of each test strain harbouring all combinations were grown at 30°C for 22 hours. This long incubation period is due to the slow growth rate of LJ2809 and its associated strains. For rapid screening, 8 µl of each overnight culture was spotted on an LB plate containing 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 mg/ml) and incubated at 30°C for 24 to 36 hours.

2.10 β-Galactosidase Liquid Assays : Quantification of *in Vivo* Protein-Protein Interactions

Experimental interactions were quantified by monitoring levels of β-galactosidase activity. Measurements were obtained using a spectrophotometric assay which takes advantage of this enzyme's ability to cleave β-D-galactosides (Miller, 1992). One such substrate is the chromogenic ONPG (*o*-Nitrophenyl-β-D-

galactosidase). It is colourless, but upon hydrolysis yields a yellow colour attributed to the presence of *o*-nitrophenol. High initial concentrations of ONPG allow for a situation where the amount of β -galactosidase present is directly proportional to the amount of *o*-nitrophenol produced. This reaction is terminated upon the addition of sodium carbonate (Na_2CO_3). This solution increases the environmental pH to a point where β -galactosidase is rendered inactive.

Cultures of LJ2809 and its isogenic counterparts harbouring all pairwise combinations were grown at 30°C for 22 hours. Cell densities were measured at OD₆₀₀ (Cary UV Spec) and 100 μl aliquots were added to previously prepared tubes containing 0.9 ml of activated Z buffer (60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$, 10 mM KCl, 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mM β -mercaptoethanol; pH 7.0) and 30 μl 0.1 % SDS.

Chloroform (3 drops) was added last. All tubes were vortexed for approximately 15 seconds before being placed in a 28°C water bath for five minutes. During this incubation, ONPG (4 mg/ml) was prepared using Z buffer without β -mercaptoethanol. Each reaction was started by the addition of 0.2 ml ONPG. The tube was shaken and returned to the bath. Once an appropriate yellow colour had appeared, the reaction was stopped by adding 0.5 ml 1M Na_2CO_3 and the reaction time noted. The optical densities at 420 nm and 550 nm were immediately recorded. The former is a measure of the *o*-nitrophenol present. The latter is required to correct for light scattering due to cell debris. β -galactosidase activity was calculated in Miller Units (MU) as per the following equation :

$$\text{Miller Units} = 1000 \times \frac{\text{OD}_{420} - (1.75 \times \text{OD}_{550})}{t \times v \times \text{OD}_{600}}$$

where

t = time of the reaction (minutes)

v = volume of the culture assayed (ml)

Three independent colonies of each strain were assayed in triplicate.

3.0 Results

3.1 Functionality of the *mut* Genes Involved in Bacterial Two Hybrid (B2H) Translational Gene Fusions

Rifampicin resistance can be used as a measure of mutagenesis. Plasmids created for use in the B2H system each contain one of three *mut* genes. Proteins translated from these exist as part of a translational fusion in conjunction with either the T18 or T25 fragment of the *B. pertussis* adenylate cyclase catalytic domain. The ability of each newly created vector to complement the mutator phenotype arising from a deficiency in its chromosomal *mut* counterpart was examined. Such a deficiency can be overcome by introducing a plasmid carrying the intact gene of interest.

The number of rifampicin resistant colonies per 10^8 viable cells observed upon introduction of pTS25 and pTL25 were comparable to those of pMQ341 (*mutS*) and pMQ339 (*mutL*) (Figure 11). This is indicative of the presence of a functional repair protein within the fusion. *mutH* in pT25 also appeared to overcome chromosomal inactivation. This effect, however, was not as complete as the one generated by wild type *mutH* (pMQ348) or by other vectors containing genes in the same fusion order (pTS25, pTL25). Genetic complementation by plasmids pTS18, pTL18 and pTH18 was not detected. High levels of rifampicin resistance indicated that MutS, MutL and MutH expressed as a fusion with the T18 fragment are unable to counteract the mutagenic effect brought about by each individual chromosomal gene failure.

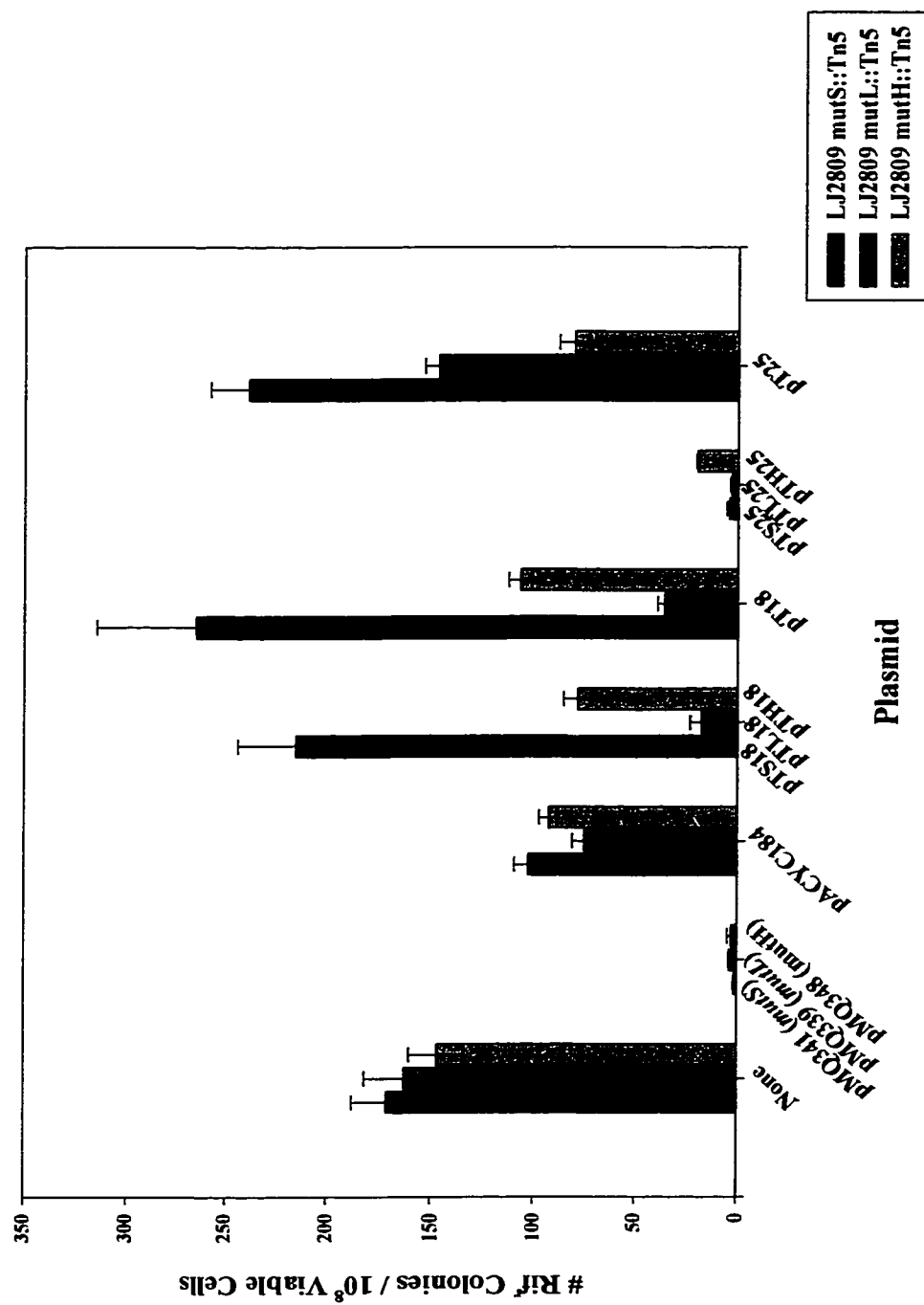


Fig. 11. Determination of B2H Fusion Protein Function Using Complementation. Each set of bars represents the effect resulting from addition of various plasmids, named underneath, to each isogenic mutant strain. Set 1: strains only, Set 2: pACYC184-based plasmids containing intact genes *mutS*, *mutL* or *mutH*, Set 3: control plasmid pACYC184, Set 4: pT18-based B2H vectors, Set 5: control plasmid pT18, Set 6: pT25-based B2H vectors, Set 7: control plasmid pT25.

3.2 Lac Reversion - A Complementation Assay for Vsr

Vsr recognizes and repairs T·G mismatches arising from the spontaneous hydrolytic deamination of 5-methylcytosines located within Dcm recognition sites. Mutations occurring at these sites within *rpoB* do not result in resistance to rifampicin. As such, genetic complementation by Vsr could not be assayed in this manner. Another alternative involved using a Vsr-deficient strain carrying an episome containing a modified *lacZ* gene, as previously described (Ruiz *et al.*, 1993; Petropoulos *et al.*, 1994)

For reasons of compatibility, only plasmids pTV25 and its associated controls were transformed into CC112-V. The Lac reversion frequencies are shown in Figure 12. Comparison of pTV25 and pT25 revealed that *vsr* contained in this gene fusion does not give rise to a repair protein in good working order. The reversion rate of pTV25 remained elevated as compared to that of pDV108, a low-copy plasmid containing *vsr* under the control of the *trc* promoter.

P90C-V also contains an episome carrying a modified *lacZ* gene. This design allows more direct monitoring of Vsr function without the need of a suppressor plasmid. All vectors containing *vsr* as part of a fusion could therefore be tested for complementation regardless of their origin of replication. The levels of Lac reversion reflect the proper functioning of Vsr and its ability to prevent CCAGG to CTAGG mutations. Although Lac reversion occurs through a C to T mutation at position 462 in more than 92% of all cases there exist other mutations that can lead to a Lac⁺ phenotype (Petropoulos *et al.*, 1994). Therefore, this strain can not only measure

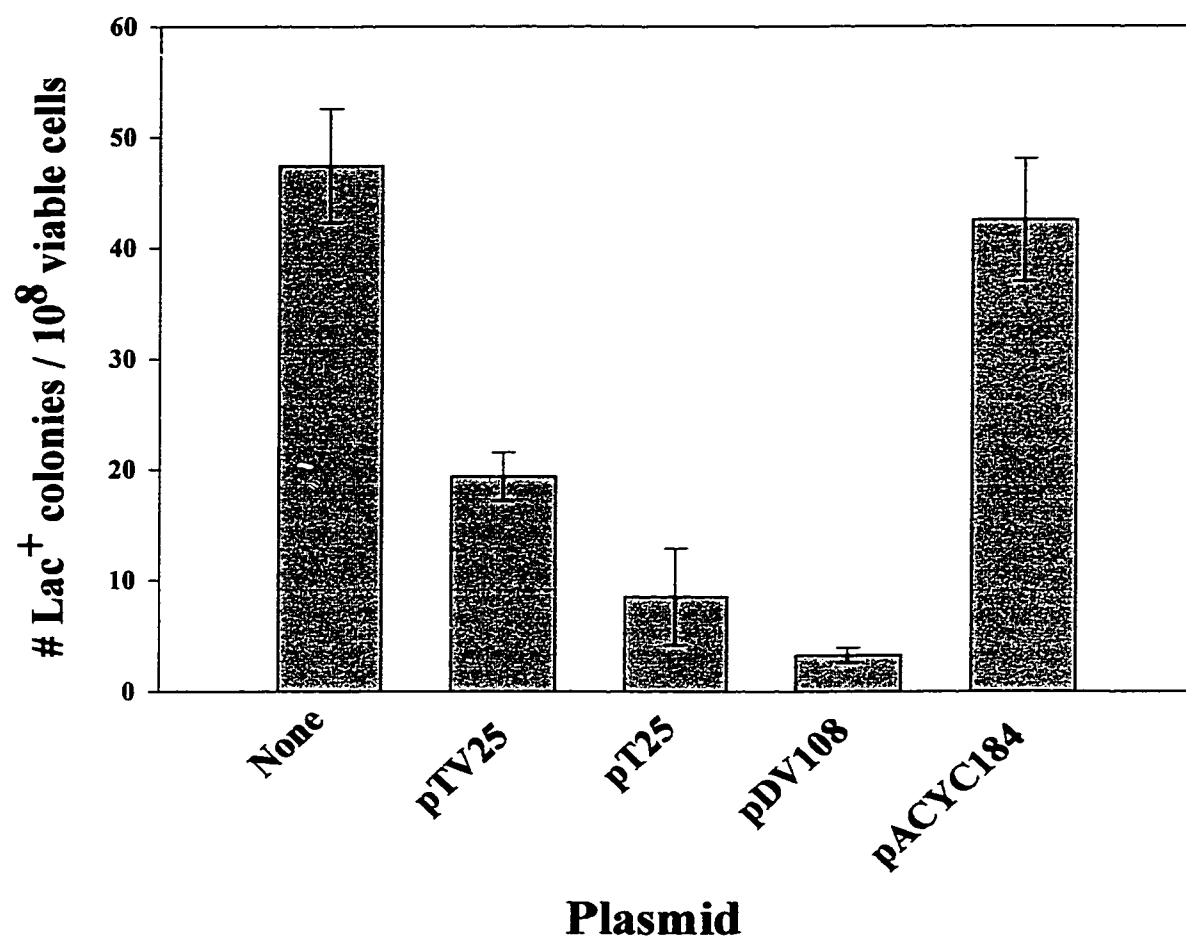


Fig. 12. Determining Vsr Function Using Complementation in CC112-V.

repair but detect mutation as well. *vsr*, in either the pT18 or pT25 vector, along with the proper controls, was introduced into P90C-V. Figure 13 shows that the number of Lac⁺ revertants for pTV25 or pTV18 does not differ from their respective controls. This suggests that Vsr is unable to undertake repair when involved in such fusions.

3.3 Qualitative and Quantitative Indications of *In vivo* Interactions in the Bacterial Two Hybrid (B2H) System

To identify potential interactions between the Mut proteins of MMR themselves as well as with Vsr, each gene was cloned into pT25 and pT18, the base vectors of the B2H system. All possible pairwise combinations were tested in LJ2809 and isogenic *mut* derivatives. Potential interactions were initially screened for on LB indicator plates containing IPTG and Xgal. The positive and negative controls consisted of the pT18zip/pT25zip and pT18/pT25 combinations, respectively. The former was previously established as interacting (Karimova *et al.*, 1998).

Cleavage of Xgal yields a blue compound. Different intensities can be correlated to differing strengths of interaction. The initial screening of all possible pairwise combinations in LJ2809 detected six positive interactions (Figure 14).

These were :

- pTS18/pTS25 • pTS18/pTL25
- pTL18/pTL25 • pTH18/pTS25
- pTH18/pTL25 • pTV18/pTL25

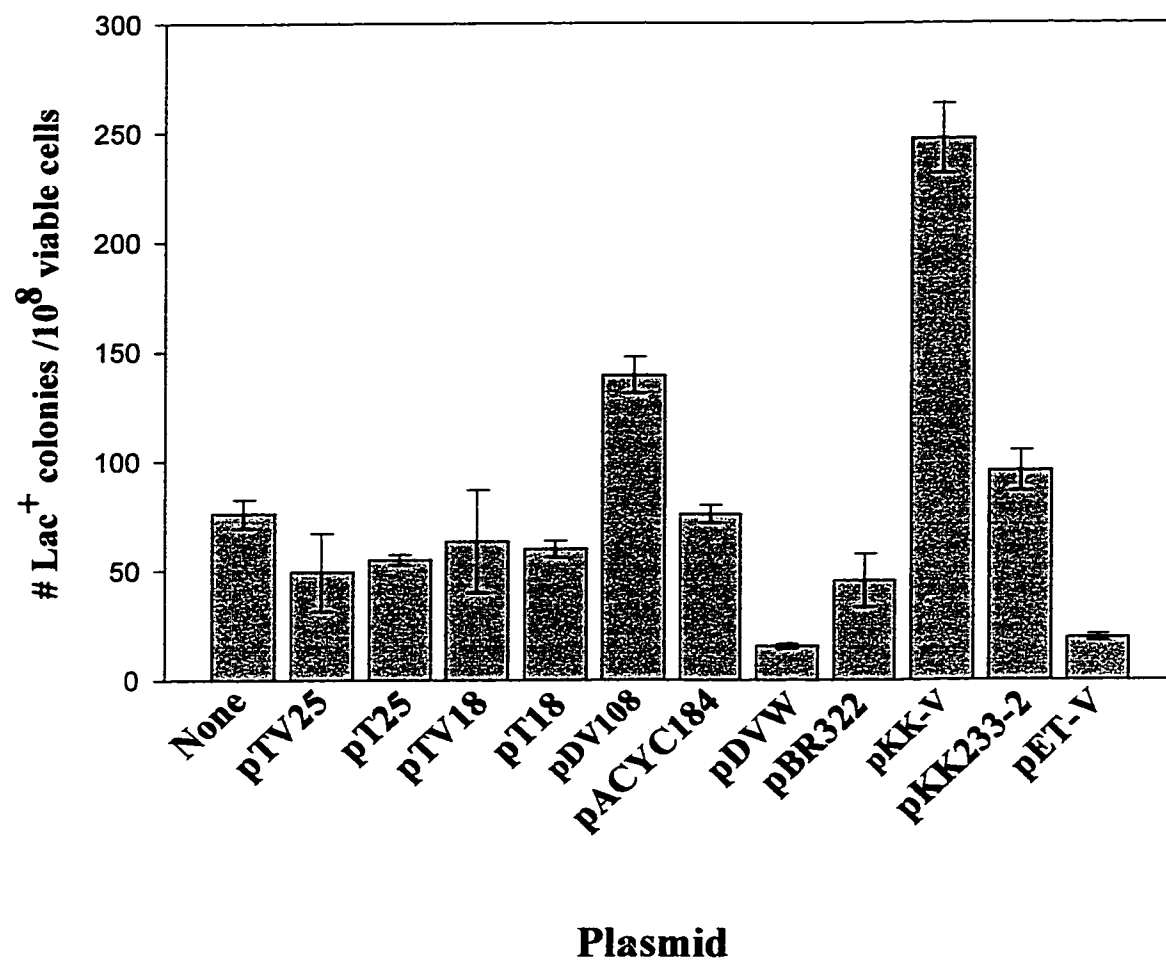


Fig. 13. Complementation of P90C-V with Vsr.

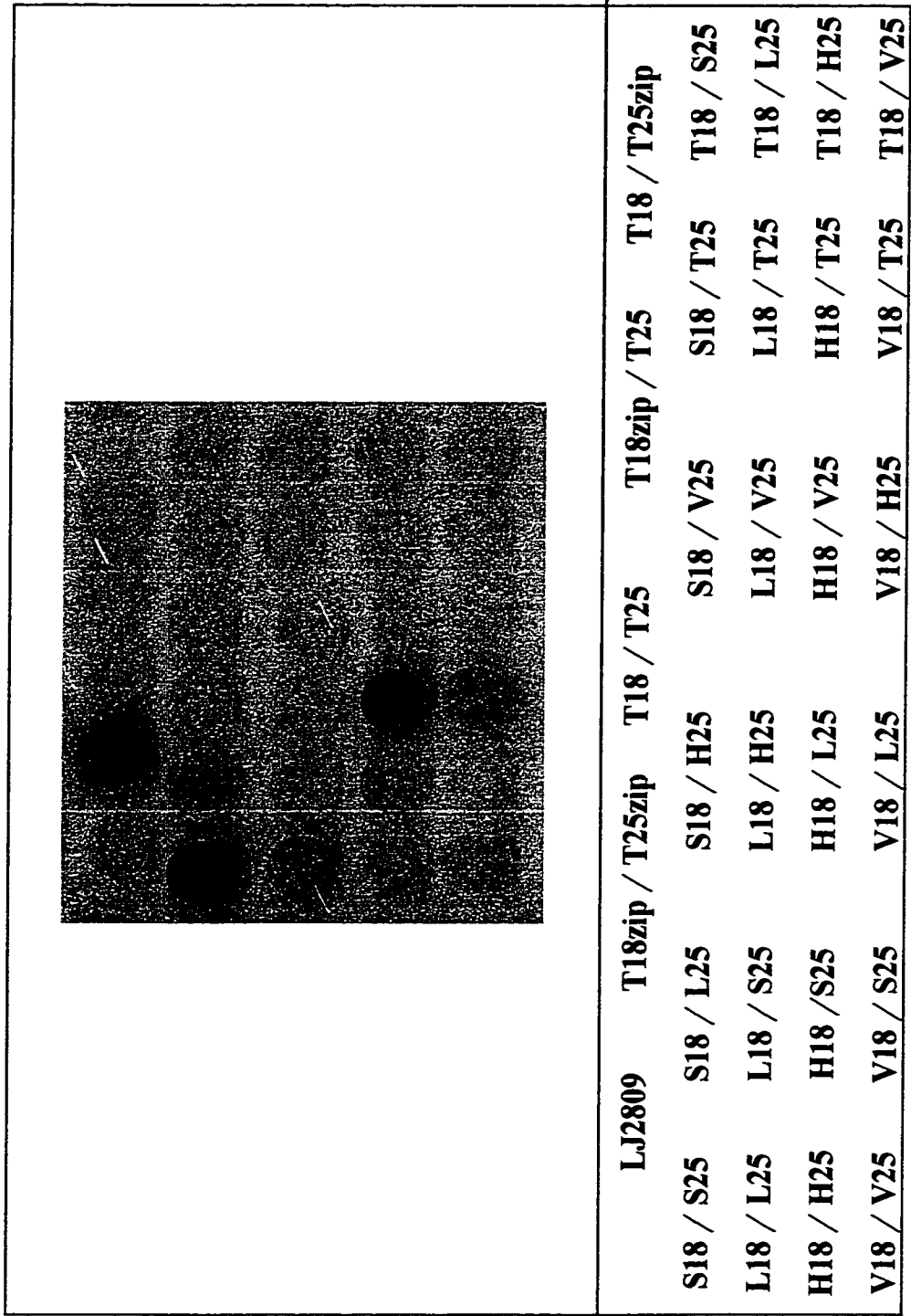


Fig. 14. Qualitative Interactions Between Mismatch Repair Proteins.

The S/L, H/S, H/L and V/L interactions did not demonstrate any reciprocity since these were observed only in the specified orientation. All controls behaved as expected. The strength of interactions was then quantified using β -galactosidase liquid assays (Figure 14).

The interactions observed can be sorted into three separate classes (strong medium and weak) based on their strengths of interaction. This may aid in defining what role they play *vis-à-vis* their partners in postreplicative repair or very short patch repair. In addition, it may shed some light on the order in which they interact during the repair process. The interaction between the two MutS monomers was by far the strongest of the six observed. It shared the same deep blue colour as the T18zip/T25zip combination and its β -galactosidase levels were estimated to be approximately 9-fold higher than background levels (Figure 15). This was comparable to the 7-fold increase seen for the positive control.

Formation of a MutL dimer was detected. Three-fold increases in β -galactosidase activity were observed with MutS/MutL and MutH/MutL associations. Interaction between the endonuclease MutH and MutL, a putative molecular matchmaker, was also evident. An indication that there may exist some overlap between two mismatch repair systems was given when Vsr interacting with MutL was discovered. These four pairings can be loosely grouped together in a second category. Finally, the lone member of the third category is the apparent MutH/MutS partnership. This last combination is the weakest and may indicate that these proteins do not normally associate together without the transient or perhaps

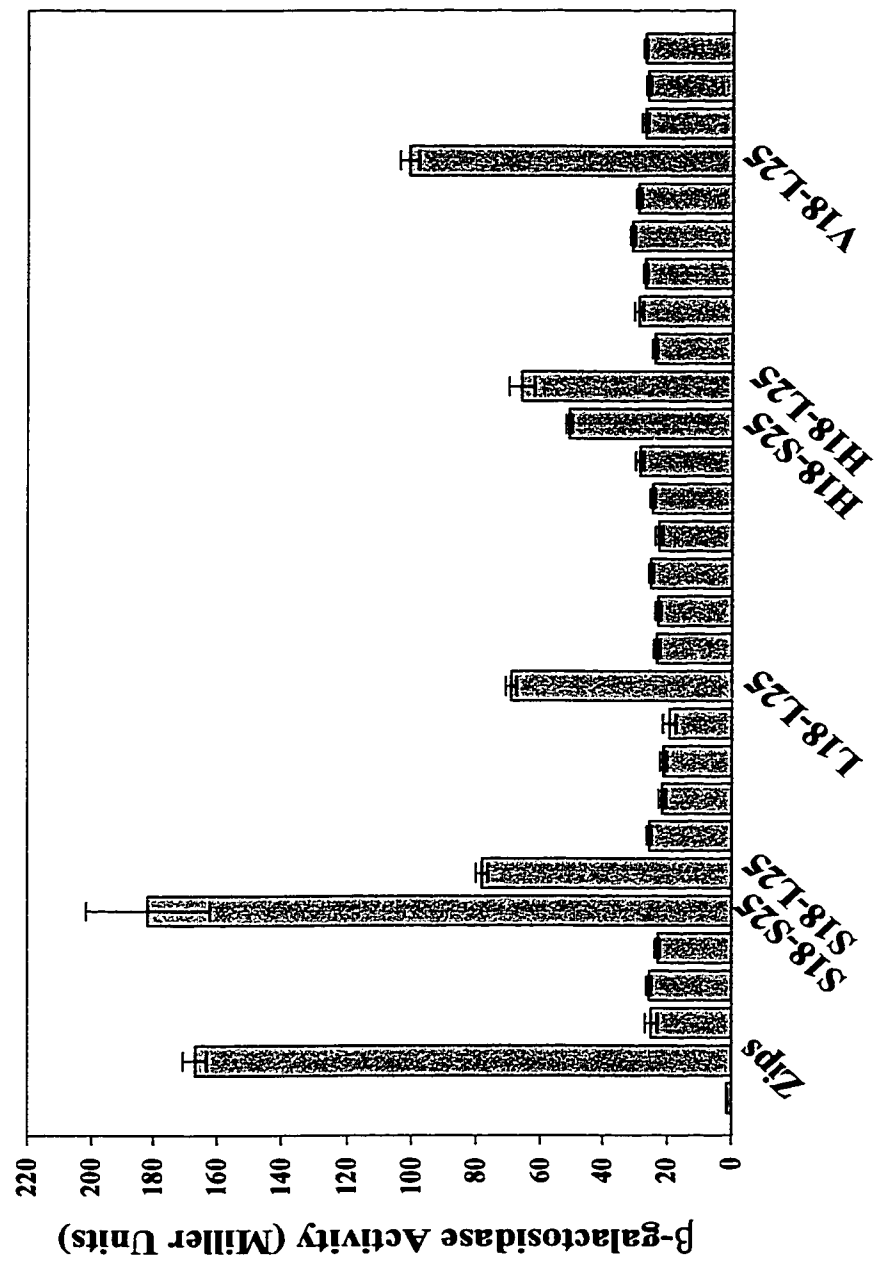


Fig.15.Quantitation of Interactions Between Repair Proteins Using Liquid β -galactosidase Assays.

more extensive involvement of another partner.

3.3.1 The Impact of Chromosomal *mut* Gene Disruption on Interactions Between Repair Proteins

MutS, MutL or MutH fused to the C-terminus of T25 retains the ability to complement strains deficient in the relevant *mut* gene. We wished to test whether interactions observed originally would still be maintained despite the absence of functional chromosomal *mut* genes. These mutagenic versions of LJ2809 were created by introducing one of three alleles, *mutS*::Tn5, *mutL*::Tn5 or *mutH*::Tn5. As in prior experiments, all possible pairwise combinations were transformed in and subsequently screened for the presence or absence of any interaction. Again, quantification was undertaken using β -galactosidase liquid assays.

In a repair-deficient *mutS*::Tn5 strain, only two interactions, those between dimers of MutL and the association of MutS and MutL, were still maintained (Figure 16). Four- and two-fold increases in β -galactosidase activity were observed for each, respectively. The reversal of the S-S interaction was the most dramatic (Figure 17). Interaction between repair systems denoted by Vsr and MutL binding was also abolished. Likewise, the H-S and H-L combinations were reversed but not to the complete extent seen with S-S or V-L. Figures 18 and 19 represent the initial phenotypic screening of all pairwise combinations following the disruption of chromosomal *mutL*. This event resulted in the disappearance of the S-L, H-S and V-L interactions. The strength of interaction of H-L appears to have diminished while the

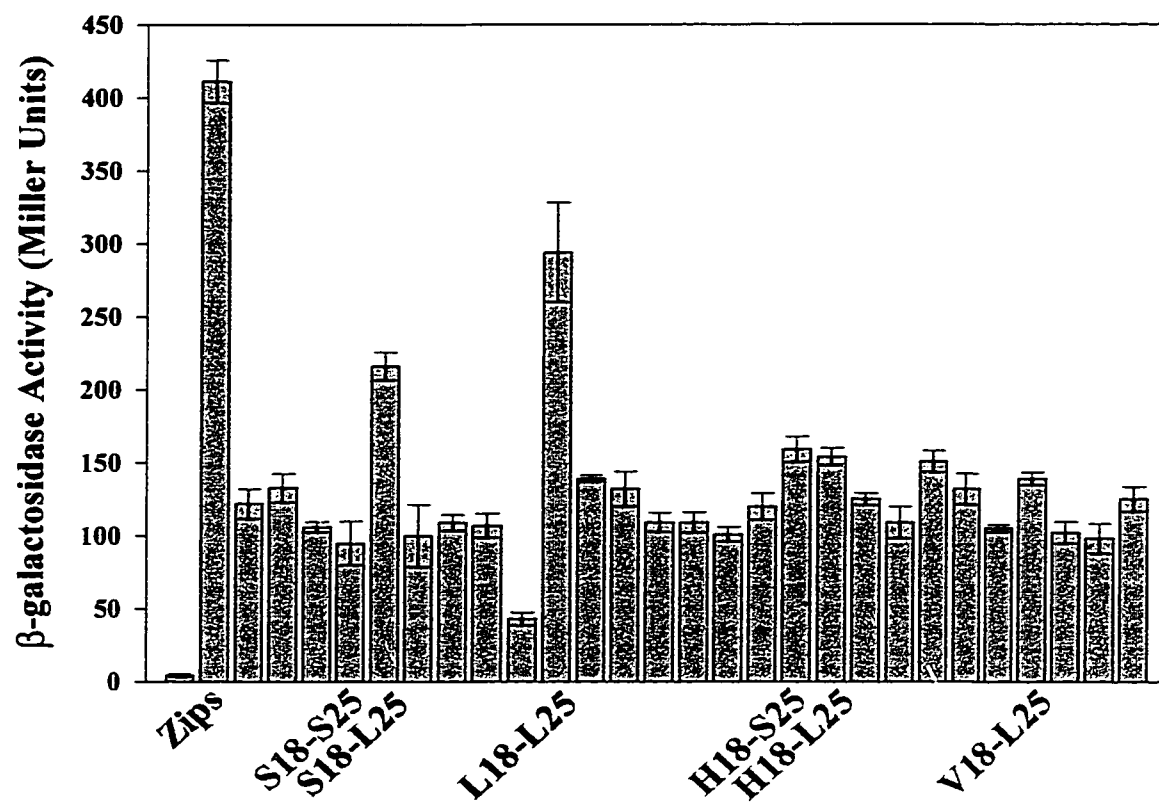


Fig. 16. Quantitation of Interactions Between Repair Proteins in a *mutS* Background Using Liquid β-galactosidase Assays.

	LJ2809 mutS::Tn5	T18zip / T25zip	T18 / T25	T18zip / V25	T18 / T25	T18 / T25zip
S+	S18 / S25	S18 / L25	S18 / H25	S18 / V25	S18 / T25	T18 / S25
S-	S18 / S25	S18 / L25	S18 / H25	S18 / V25	S18 / T25	T18 / S25
S+	L18 / L25	L18 / S25	L18 / H25	L18 / V25	L18 / T25	T18 / L25
S-	L18 / L25	L18 / S25	L18 / H25	L18 / V25	L18 / T25	T18 / L25

Fig. 17. Qualitative Interactions of Repair Proteins in a *mutS* Background.

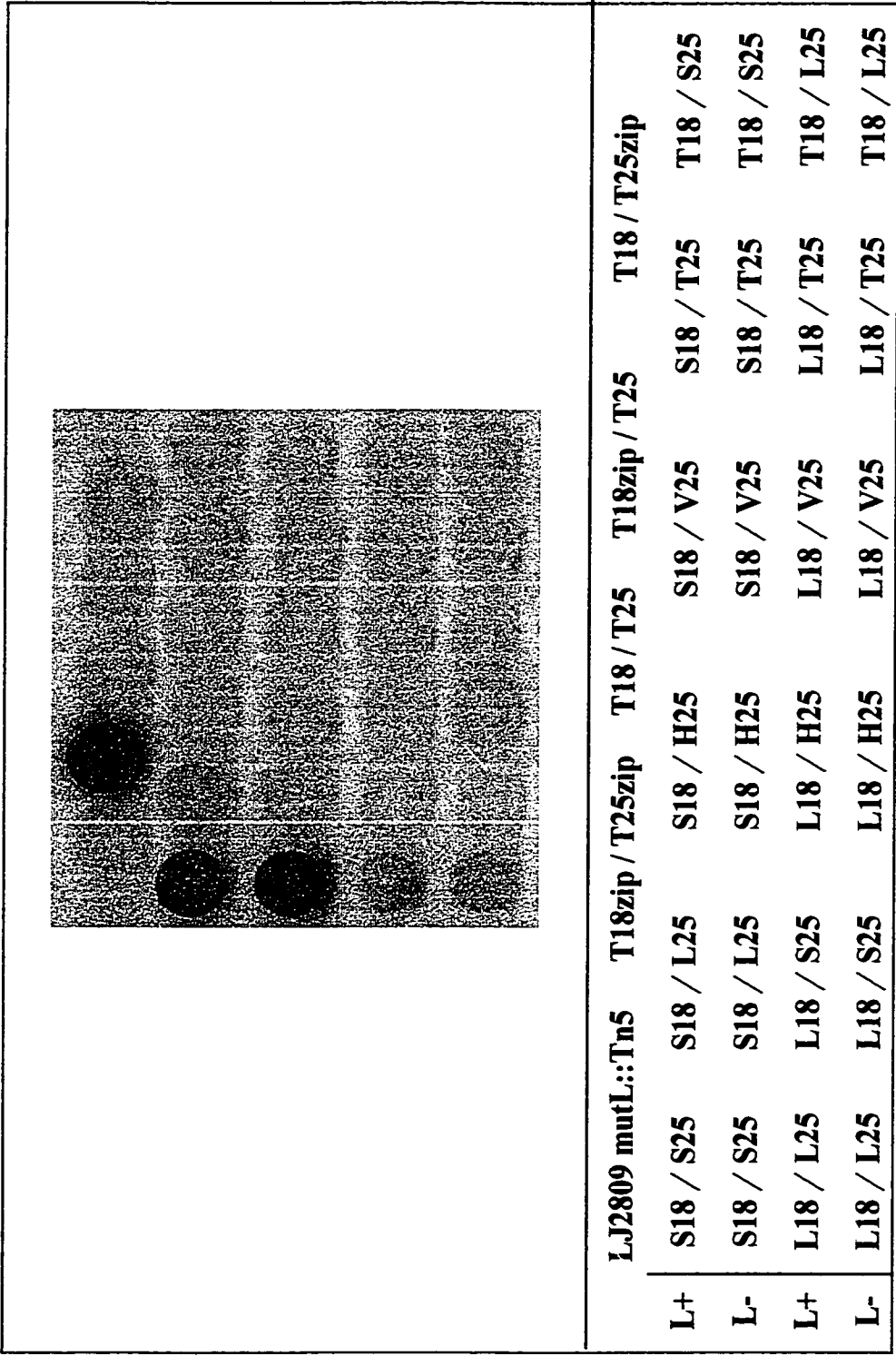


Fig. 18. Qualitative Interactions of Repair Proteins in a *mutL* Background.

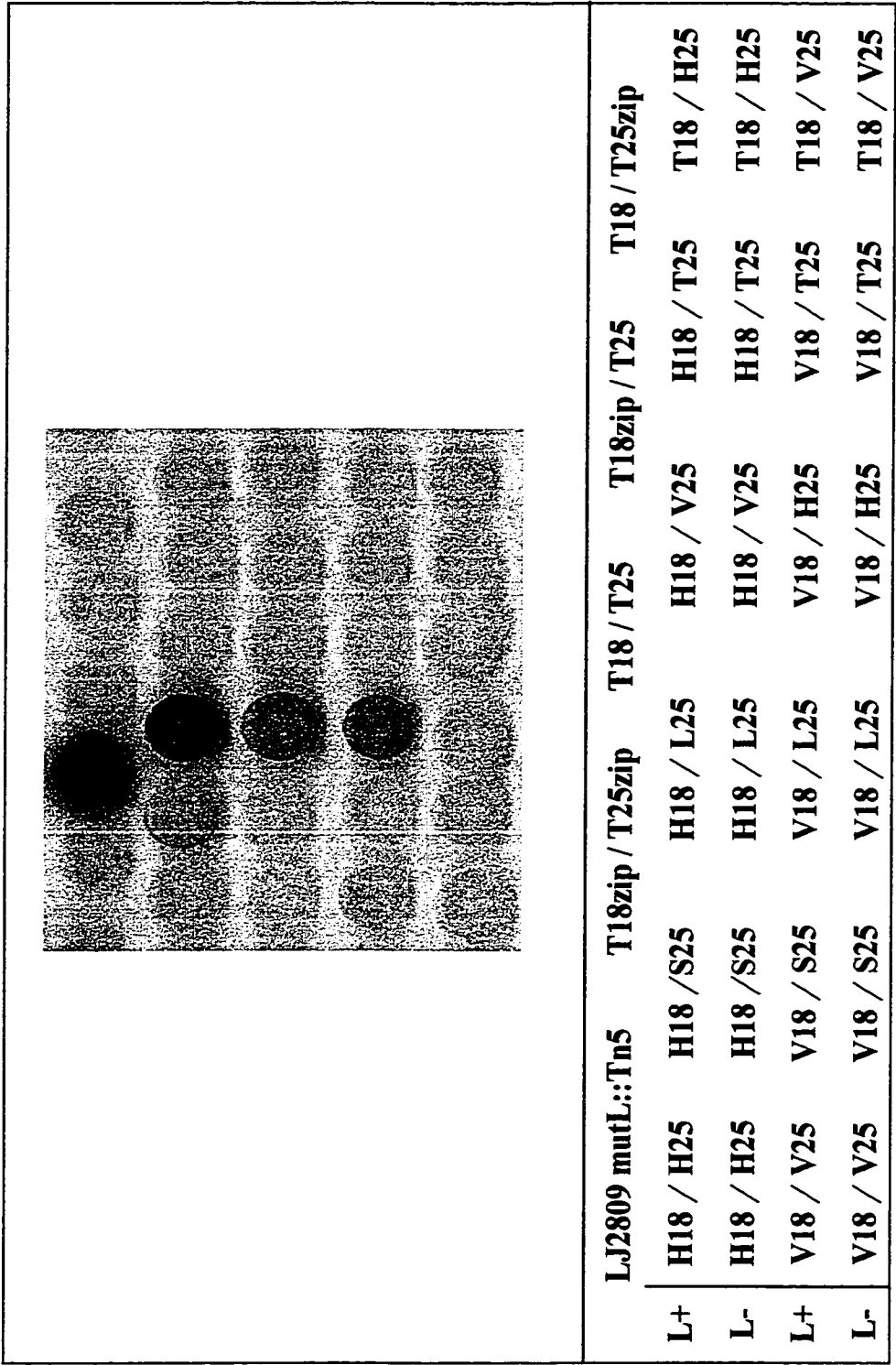


Fig. 19. Qualitative Interactions of Repair Proteins in a *mutL* Background (Continued).

formation of MutS or MutL dimers seem to have been largely unaffected. These results were for the most part well supported by subsequent β -galactosidase assays (Figure 20). The sole discrepancy lies with the L-L pairing. The spectrophotometric test appears to support the idea that these no longer associate.

Finally, the presence of the *mutH::Tn5* allele in LJ2809 seemed to counteract all positive associations with the exception of V-L (Figures 21 and 22). Quantification of these interactions supported the phenotypic screen (Figure 23).

3.4 Interference by Vsr and its Effects on Interactions Between *dam*-Directed Mismatch Repair Proteins

Evidence has been presented to the effect that the efficiency of Very Short Patch repair is greatly diminished in the absence of MutL or MutS. Furthermore, MutL has been shown to greatly enhance Vsr activity. Therefore, it is possible that the reciprocal presence of Vsr may hinder the smooth running of MMR by interrupting cooperative activities between some of its constituents. Identification of any such disruptions was achieved by creating vectors that contained *vsr*, under the control of a strong synthetic promoter, at an alternate site within pT18-based B2H vectors. The three vectors in question already contained either *mutH*, *mutL* or *mutS* in an in frame translational gene fusion with the portion of the adenylate cyclase gene corresponding to the T18 end of the catalytic domain.

Figures 24 and 25 clearly show that the presence of excess Vsr abolishes the L18-L25 interaction as well as the union between the endonuclease MutH and the mismatch recognition component of postreplicative repair, MutS (H18-S25).

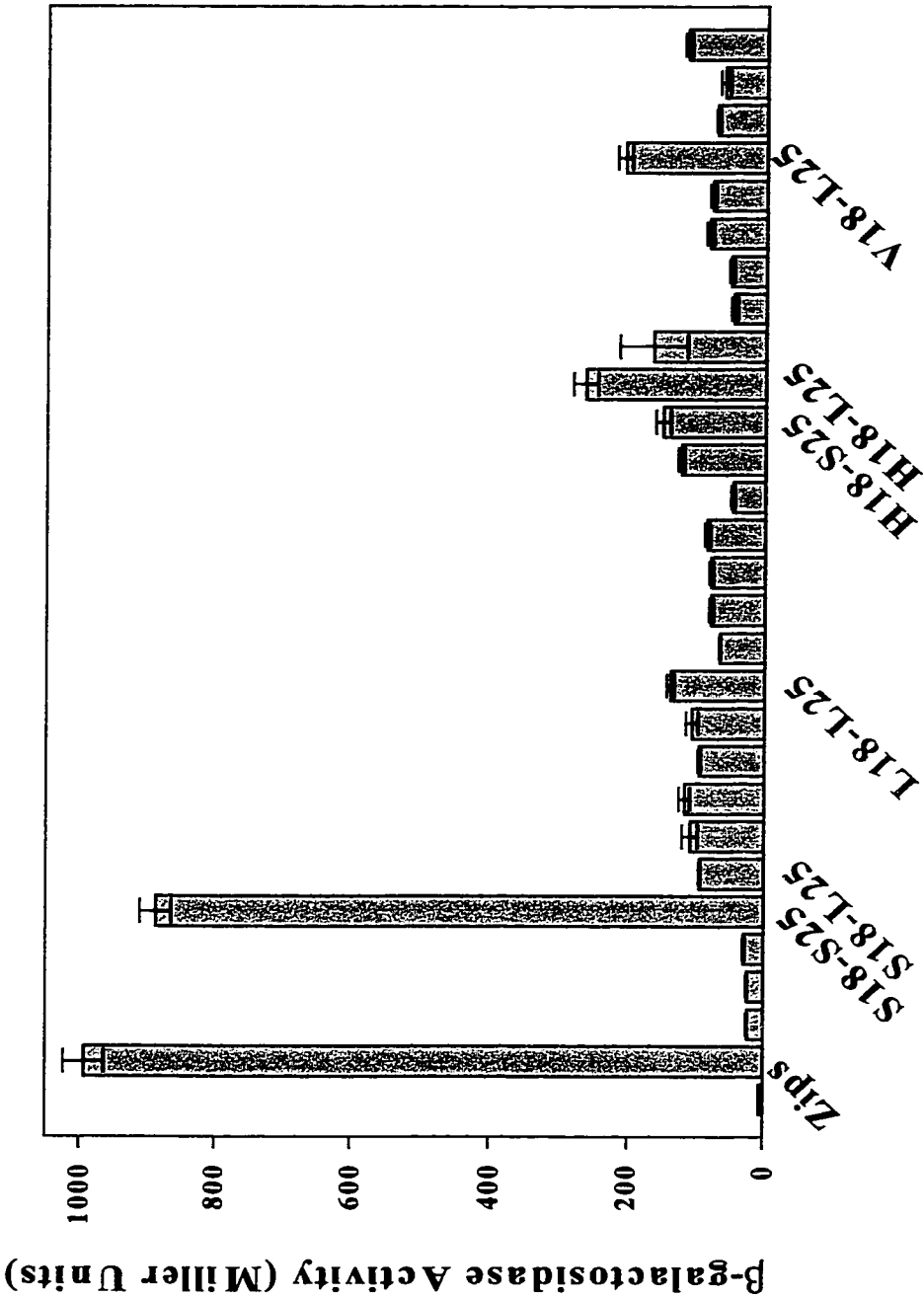


Fig. 20. Quantitation of Interactions Between Repair Proteins in a *mutL* Background Using Liquid β -galactosidase Assays.

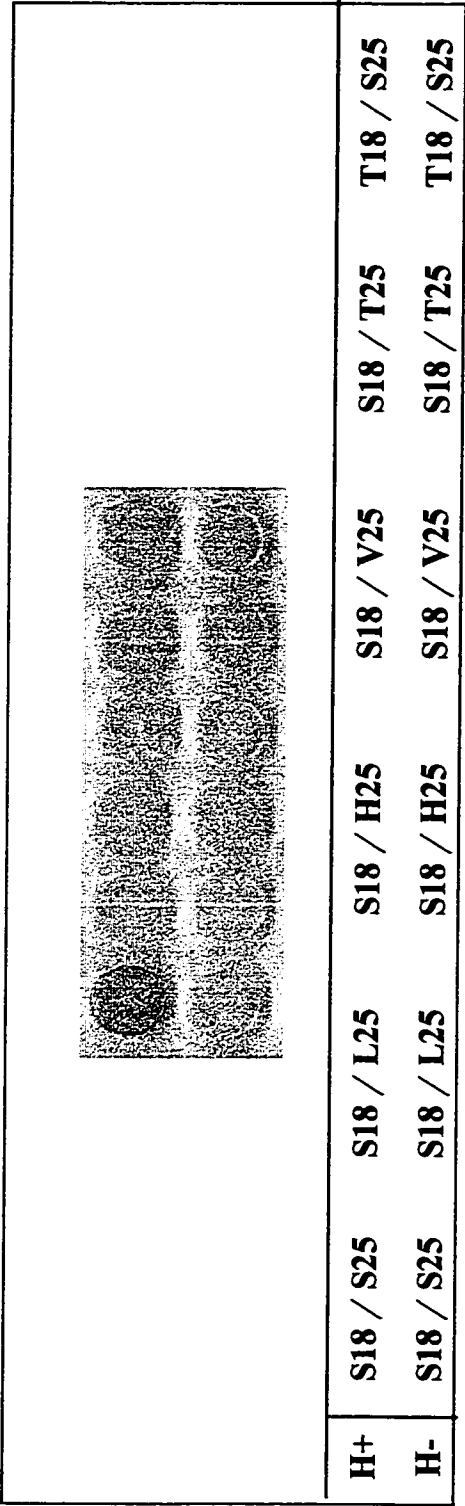


Fig. 21. Qualitative Interactions of Repair Proteins in a *mutH* Background.

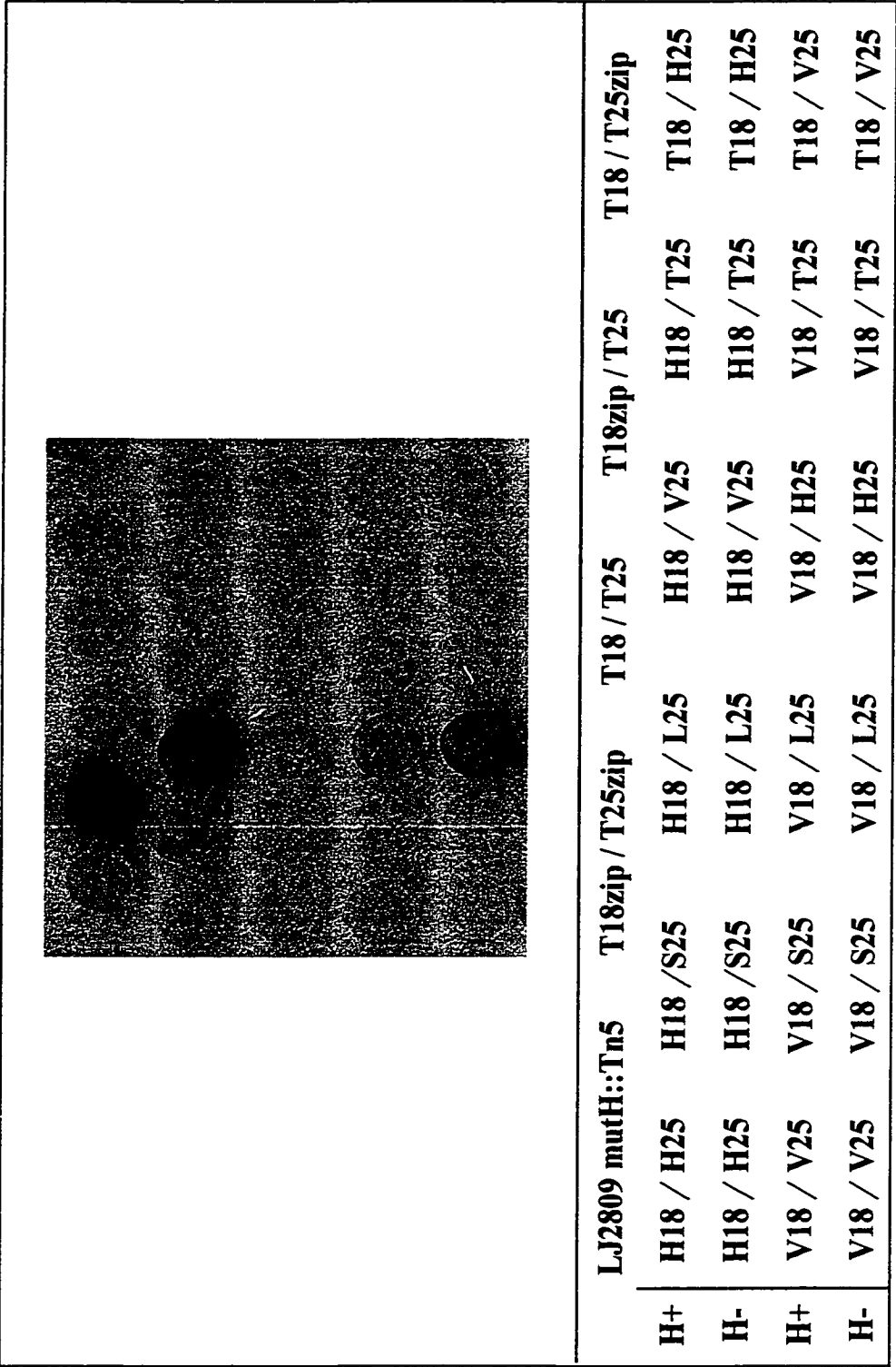


Fig. 22. Qualitative Interactions of Repair Proteins in a *mutH* Background (Continued).

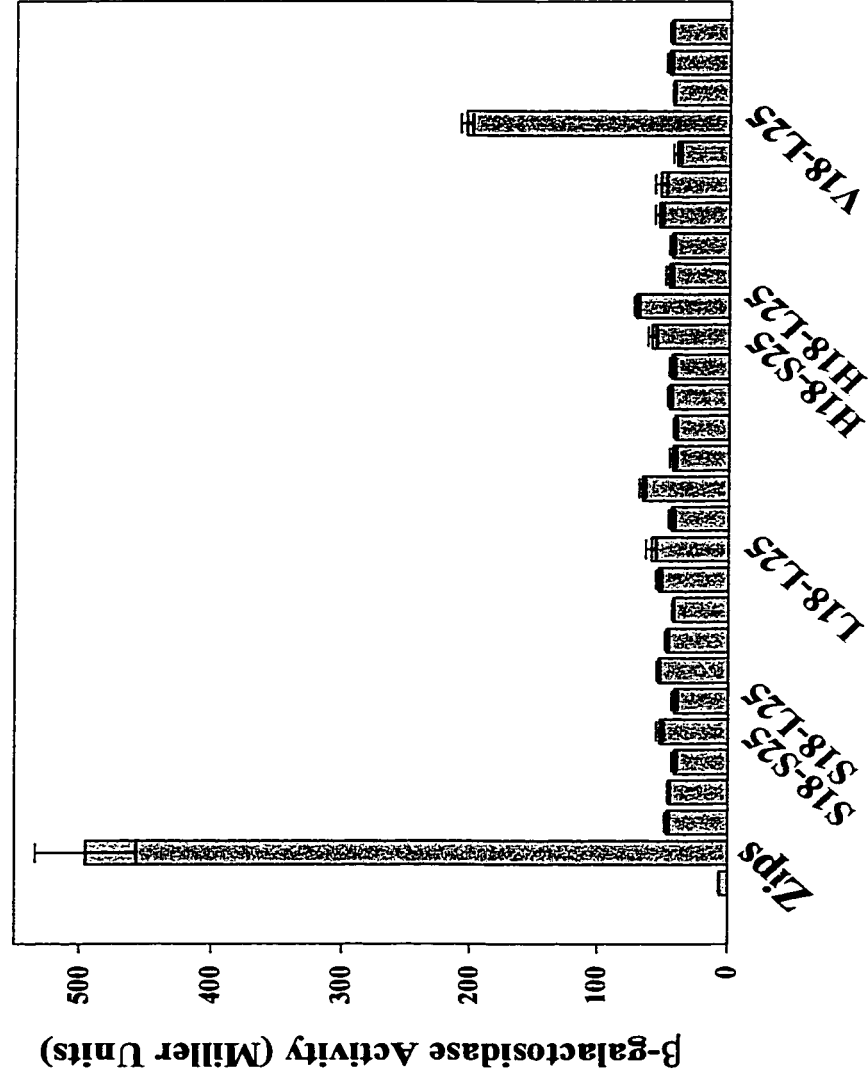


Fig. 23. Quantitation of Interactions Between Repair Proteins in a *mutH* Background Using Liquid β -galactosidase Assays.

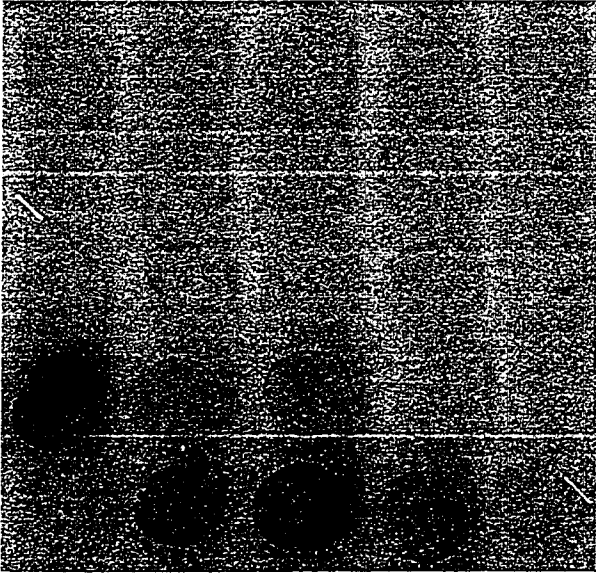
				
LJ2809	T18zip / T25zip	T18 / T25	T18zip / T25	T18 / T25zip
S18 / S25	S18 / L25	S18 / H25	S18 / V25	S18 / T25
SV18 / S25	SV18 / L25	SV18 / H25	SV18 / V25	SV18 / T25
L18 / L25	L18 / S25	L18 / H25	L18 / V25	L18 / T25
LV18 / L25	LV18 / S25	LV18 / H25	LV18 / V25	LV18 / T25

Fig. 24. Qualitative Indications that Vsr Interferes With Methyl-directed Mismatch Repair.

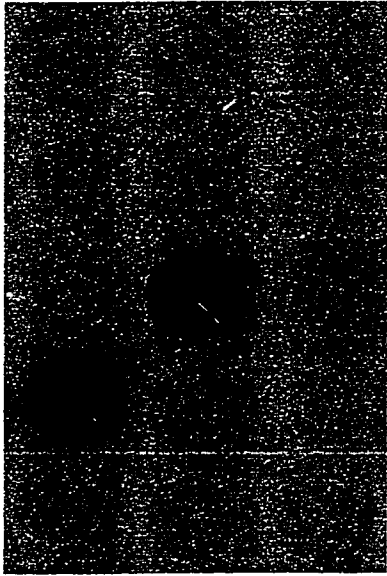
					
LJ2809	T18zip / T25zip	T18 / T25	T18zip / T25	T18 / T25zip	
H18 / H25	H18 / S25	H18 / L25	H18 / V25	H18 / T25	
HV18 / T25	HV18 / S25	HV18 / L25	HV18 / V25	HV18 / T25	

Fig. 25. Qualitative Indications that Vsr Interferes With Methyl-directed Mismatch Repair (Continued).

Although Figure 25 portrays a significant weakening of the association between MutH and MutL (H18-L25), β -galactosidase activities, which represent a more precise measure of interaction, indicate the complete extinguishing of this combination (Figure 26). Reversal of the other interactions, first identified by visual phenotypic changes, was also confirmed by spectrophotometric tests. The enzyme activity associated with each of these interactions was on par with basal levels. The S-S grouping was the sole positive candidate to remain unaffected by the presence of Vsr. This condition was reflected in the form of an almost 9-fold increase in β -gal activity as compared to background.

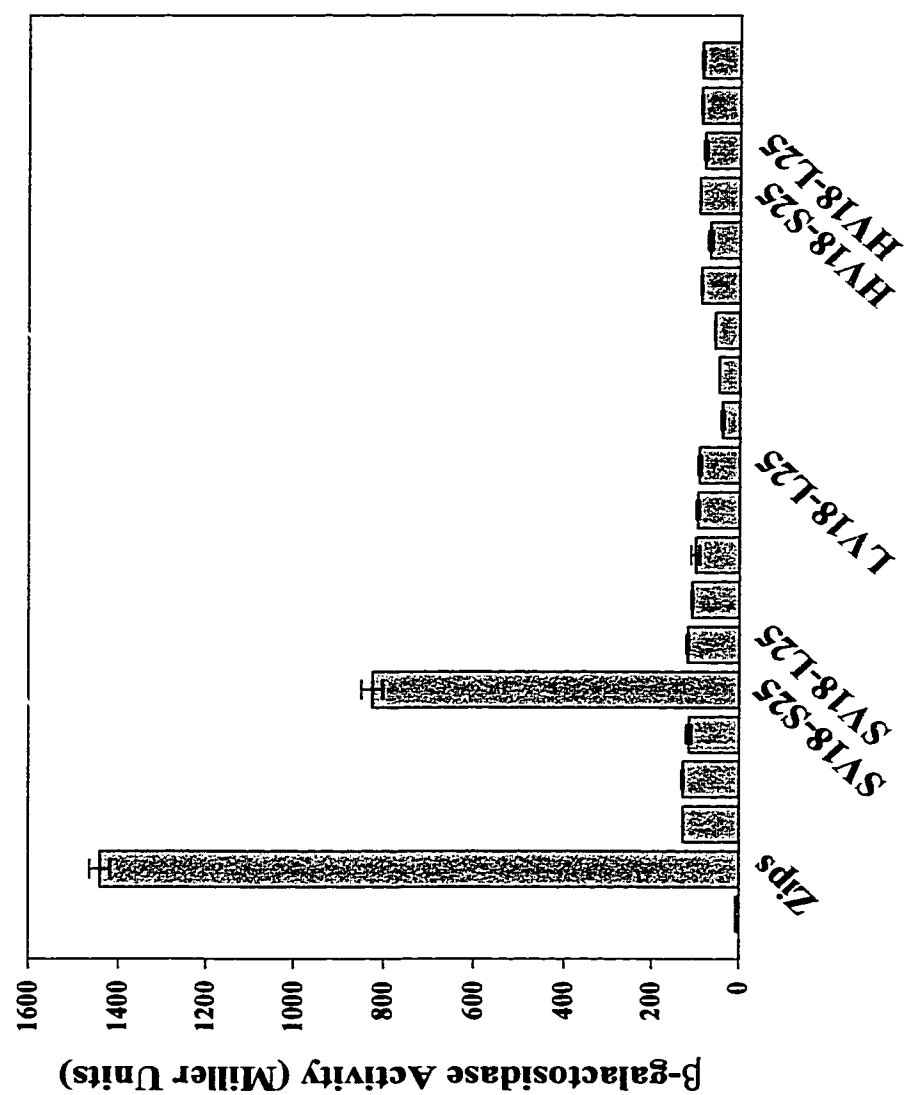


Fig. 26. Quantitation of Interactions Disrupted By Overabundance of Vsr Using Liquid β -galactosidase Assays

4.0 Discussion

The past fifteen years have seen a great deal of time and effort invested in the study of *Escherichia coli* DNA repair systems, more specifically methyl-directed mismatch repair. Extensive analyses have led to a model outlining its general operation. Although the essential features of MMR have been revealed, the more specific nature of each step in this critical process still requires clarification.

MutH, MutL and MutS are integral to the proper functioning of post replicative repair. Moreover, the last two have been implicated in the efficient endonucleolytic functioning of Vsr, primary player of very short patch repair. This study made use of the bacterial two hybrid (B2H) system in an attempt to further elucidate the role that each of these proteins plays relative to one another. The B2H system is based on the premise that two putatively interacting proteins, when fused to either fragment of the *B. pertussis* catalytic domain of adenylate cyclase, will reconstitute its catalytic activity. Synthesis of cAMP, followed by its subsequent association with CAP, allows the transcription of a diverse number of sugar operons such as maltose or lactose.

Initial screening revealed six positive interactions (Figure 14), including that between MutS and MutL. Lahue and his colleagues (1989) provided earlier *in vitro* demonstrations of the need for MutS, MutL and MutH involvement, as well as a number of other proteins, in the proper functioning of *dam*-directed repair. This contributed to the establishment of the current model for MMR which contends that MutS triggers repair by binding to the mismatch, an action followed soon after by the

ATP-dependent binding of MutL (Grilley *et al.*, 1989). This physical interaction was observed in footprint analyses (Grilley *et al.*, 1989), electron microscopy studies (Allen *et al.*, 1997) and immunoblotting experiments (Wu & Marinus, 1999). Therefore, it was not surprising that such an interaction was observed in the B2H system.

Formation of MutS and MutL homodimers was observed, confirming previously published reports that both of these exist in such a state (Su and Modrich, 1986; Allen *et al.*; Grilley *et al.*, 1989). Genetic complementation studies (Figure 11) provided evidence that S25 fusion proteins were repair proficient. This was achieved by virtue of their ability to compensate for the lack of chromosomal MutS. Such was also the case for L25 fusion proteins (Figure 11). The existence of active S25 or L25 homodimers was further substantiated upon the discovery that S-S and L-L interactions disappear in *mutS* and *mutL* strains, respectively (Figures 16 and 20). Furthermore, MutS and MutL cease to associate in a MutL-deficient strain while the MutH-MutS link vanishes in a *mutS* background. All of the above taken together point a finger to the more than likely possibility that these dimers are being recruited by the normal MMR machinery and thus are unavailable to form interactions with their respective partners.

MutL, in formation with the MutS/mismatch-containing DNA complex, can bind and activate the endonucleolytic abilities of MutH (Hall *et al.*, 1999). The completion of this triumvirate leads to MutH nicking the undermethylated strand 5' of the d(GATC) site located closest to the mismatch. This physical interaction was

another one of the original six first seen using the B2H system (Figure 14) and confirms yet another step in postreplicative repair.

Another interaction that holds the attention is the weak but definitive association between the recognition component, MutS, and the endonuclease MutH, an affiliation never having been witnessed before (Figure 14). There is conflicting evidence concerning the role MutS plays in the stimulation of the MutH endonuclease activity. On the one hand, MutS has previously been demonstrated to have no effect on this activity (Hall & Matson, 1999) while another report states that the presence of MutS and ATP aids MutL to activate MutH (Ban & Yang, 1998). The MutS union with MutH, albeit weak, signals that MutS may contribute an added level of stability to the MMR complex, further supplementing that provided by MutL. Such assistance would be of even greater importance if MutL was found to leave the complex upon MutH activation. This may be a distinct possibility since MutL has been found to bind (Hall *et al.*, 1998) and activate (Yamaguchi *et al.*, 1998) DNA helicase II. Further to this, both MutH and the helicase bind MutL at its C-terminus ((Hall *et al.*, 1998; Hall & Matson, 1999), carrying with it the implication that releasing one would enable the binding to another.

Although completely novel, the H-S interaction can be afforded quite a bit of weight in light of the fact that neither this combination nor the S-L alliance or the homodimerization of MutS and MutL was detected in the yeast two hybrid system (Figure 27). Furthermore, this potential H-S interaction has not been picked up using traditional biochemical methods. These facts establish the validity of the bacterial two

hybrid system as a sensitive tool that can be used for rapid and reliable *in vivo* screening of potentially interacting proteins.

Interestingly, disabling the chromosomal *mutL* gene leads to the disappearance of the H-S interaction (Figure 20). This lends support to the proposal that MutL functions as a molecular matchmaker (Sancar & Hearst, 1993), as UvrA does in the nucleotide excision repair pathway. Molecular matchmaking is the ability of a protein, through the use of ATP hydrolysis, to promote the stable joining of two normally individual but compatible entities by eliciting a conformational change from one or both components. Sancar and Hearst (1993) outlined the five characteristics below as a measure of whether a protein possesses the qualities required to be classified as a molecular matchmaker.

1. The affinity of the two entities for each other must be minimal in the absence of the matchmaker.
2. The matchmaker must be able to promote a stable complex between the individuals.
3. At least one of the three components of the complex must be either an ATPase or an ATP binding protein.
4. The presence of the matchmaker within the complex must cause conformational change but not a permanent modification of either component.
5. The matchmaker must dissociate from the newly formed complex so as to allow it the latitude to perform its function.

MutL possesses four of these, strengthening the hypothesis that it may be a

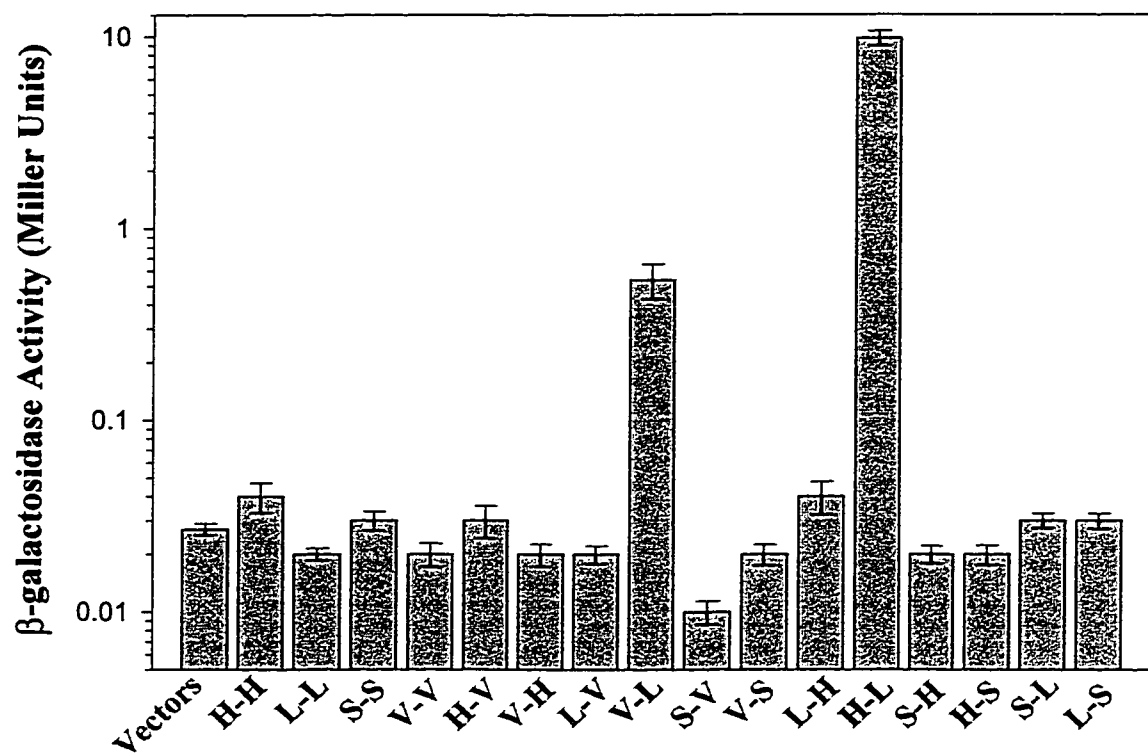


Fig. 27. Interactions between repair proteins in the yeast two hybrid system (Doiron & Cupples, unpublished).

matchmaker. It is not possible to know at this point in time whether MutL fulfills the last characteristic since its fate after activating MutH is still largely undetermined. Physical removal of the offending nucleotide is set into motion upon cleavage of the undermethylated strand by MutH. Unwinding of the DNA by DNA helicase II, stabilization of the strand by the single-strand binding protein and exonucleolytic degradation by the appropriate enzymes follows. These events notwithstanding, initial MutH activation requires its participation in a MutL-MutS-DNA complex with MutL serving to ignite its endonucleolytic activity. Bearing this in mind, the abolition of all interactions between mismatch repair proteins upon disruption of MutH production was quite unexpected (Figure 23). If MutH was unavailable, one would have to ponder the fate of the MutS-MutL complexes formed beforehand. Do they dissociate after a certain period of time, perhaps after the window of opportunity for MMR to work has elapsed, or do they possibly linger on the DNA, unable to extricate themselves ? The latter explanation is an attractive one. If chromosomal MutS and MutL were consistently unable to remove themselves from the DNA, a shortage of these would soon result. Plasmid-borne MutS25 or MutL25 would temporarily be able to make up for this shortfall though it would only be a matter of time before these would be titrated out as well. Sequestration of S25 and L25 homodimers in the initial repair complexes would make them unavailable for interaction with their respective partners.

This hypothesis stands to reason since a similar scenario was observed involving retons scattered within the *Escherichia coli* genome (Maas *et al.*, 1996).

Retrons are genetic elements encoding structures composed of single-stranded DNA covalently linked to RNA named msDNAs (Maas *et al.*, 1994). These structures contain inverted repeats causing the formation of a stem-loop containing at least one mismatch. High concentrations of msDNAs, produced when retons are carried on multicopy plasmids, are mutagenic (Maas *et al.*, 1996). This, however, is not usually the case since the quantity of msDNAs produced by retons incorporated in the chromosome is minute, thus limiting its mutagenic power. Comparison of the mutation rate inherent to retron-containing strains or *mutS* strains revealed them to be equivalent. Maas and his colleagues speculated that this phenomenon was due to the titrating out of MutS since addition of excess MutS on a plasmid (pMQ341) inhibited mutagenesis. Most important is the lack of d(GATC) sites, which are recognized by MutH, within the structure. This would ensure the constant binding of MutS to the DNA, an action consequently leading to a never-ending cycle of mismatch repair initiation without possibility of completion.

Several lines of evidence have previously alluded to the existence of repair system overlap. Lieb (1987) demonstrated that peak efficiency of Vsr is dependent upon the presence of MutS and MutL. Drotschmann *et al.* (1998) emphasized the idea that MutL likely stimulates Vsr by enhancing its DNA binding abilities, perhaps in a catalytic manner. The most straightforward conclusion would be that one or both MMR proteins must bind to Vsr. Preliminary evidence indicated that MutL, but not MutS, does indeed interact with Vsr in the yeast two hybrid system (Figure 27, Doiron & Cupples, unpublished). This was further substantiated once the same

interactions were observed in the B2H system (Figure 15). Establishing that Vsr and MutL bind lays the groundwork for the idea that direct contact is necessary so as to allow MutL or Vsr to carry out its role.

Ordinarily, only small quantities of Vsr are produced in a cell (Dar & Bhagwat, 1993). It is interesting then that the need for MutL and MutS can be bypassed if additional amounts of Vsr are introduced (Sohail *et al.*, 1990). However, MutL and MutS are most likely required because overexpression of Vsr is mutagenic (Doiron *et al.*, 1996). The mutation spectrum of a strain overexpressing Vsr is akin to that of a strain deficient in MMR, leading to the suggestion that overabundance of Vsr saturates MMR by disabling some of its components. The need for balancing levels of cellular Vsr, so as to maintain the line between repair and mutation, is exemplified by genetic complementation experiments involving the aforementioned endonuclease (Figure 13). Low levels of Vsr, as provided by either pET-V or pDVW, result in a low number of Lac⁺ revertants produced. This minimal amount intimates that repair is taking place. Mutation is stimulated by an overabundance of Vsr. Such an event is witnessed in the presence of the low-copy plasmid pDV108, which produces excessive amounts of the protein, and is accompanied by a high rate of Lac reversion. This remarkable aspect is even more flagrant in the presence of pKK-V, a high-copy plasmid containing *vsr* under the control of the strong *trc* promoter.

Interference studies provided evidence that Vsr hinders MMR repair (Figure 26). Homodimerization of MutL was nonexistent in the presence of elevated levels

of Vsr while all interactions involving MutL were disrupted. This increases the likelihood that MutL may indeed act as a molecular matchmaker. Such a role would make it the most likely candidate for incapacitation by Vsr. The crystal structure of MutL may give some indication as to how Vsr achieves this. Recall that activation of MutL occurs in four steps: ATP binding, loop ordering, dimerization and ATP hydrolysis. Ordering of the loops within the N-termini of its subunits involves the realignment of over 120 residues (Ban *et al.*, 1999). This step is potentially very slow and could represent the rate-limiting step of the activation process. Since the C-terminus of Vsr binds the N-terminus of MutL (Doiron & Cupples, unpublished), release of MutL by Vsr might result in the dissociation of the N-termini of MutL. Dissociation makes it unavailable to partake in its role within MMR until such time as the N-termini have reassociated, recreating a catalytically active enzyme.

MutL exerts its effects directly on Vsr. MutS, on the other hand, does not bind to Vsr, yet its presence is obviously called for. A possible role for MutS may be as a “carrier”, delivering MutL to Vsr. How is this possible? First, hydrolytic deamination of 5-methylcytosines would be expected to occur mainly in quiescent (non-replicating) cells. As such, short patch repair would be the system of choice to repair these lesions. Although MMR does not occur in these cells, a minimal amount of MutS is still present while the quantity of MutL is constant (Feng *et al.*, 1996). The unchanging amount of MutL is yet another point in favour of the idea that MutL is responsible for the coordination of two separate repair systems. Second, S-L interactions are not disabled in a *mutS* background (Figure 16). One may conclude

that MutS18 (composed of S18 monomers), although unable to fulfill its repair obligations, retains the ability to bind MutL. From this, one can hypothesize that MutS may be able to bind MutL without the need of a mismatch. Third, Bjornson *et al.* (2000) showed that ATP binding to MutS promotes oligomerization of MutS dimers in a concentration-dependent manner. The existence of some heterogeneity within the various ATP and DNA binding sites within the complex was proposed by the same group. Furthermore, it was also shown that ATP hydrolysis by MutS is induced upon binding to a homoduplex. Fourth, ATP hydrolysis by MutS has previously been linked to conformational changes undergone by MutS (Allen *et al.*, 1997). Such conditions, combined with the energy derived from hydrolysis, may allow the loose docking of MutL and “transport” of MutL along the DNA in search of Vsr. Once in the vicinity of Vsr, MutL would dissociate from MutS. In conclusion, if the presence of MutS was diminished, MutL would have more difficulty finding Vsr. This might explain the decreased strength of interaction seen between MutL and Vsr when chromosomal MutS production was abolished (Figure 16).

The majority of proteins exert their effects by interacting with other proteins. Pathways such as those for DNA repair are networks dependant upon cooperation between proteins. The advent of two hybrid systems has revolutionized the way these interactions are discovered and subsequently studied. This bacterial two hybrid (B2H) system is an outgrowth of the original hybrid method pioneered by Fields and Song. One of its major advantages is that it allows the study of *Escherichia coli*

protein interactions within their own natural environment. Further, it is attractive due to its simplicity and ease of use.

Methyl-directed mismatch repair (MMR) is analogous to a group of interrelated elements, each specifically designed and choreographed to work together as a coherent entity. The importance of studying the structure and function of MMR is highlighted by increases in mutation rate ranging from one hundred to one thousand fold observed if this repair system is not functioning properly. In humans, defects in MMR have been linked to a predisposition towards cancer. As demonstrated by this study, the B2H system represents an efficient way to analyze interactions. Any information obtained can lead to the establishing of a functional model. This, in turn, may provide useful information applicable to other repair systems, human or otherwise.

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