DEVELOPMENT OF A MUTANT STRAIN OF *ESCHERICHIA COLI* FOR MOLECULAR CLONING OF HIGHLY METHYLATED DNA

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BY

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To the Dean for Graduate Studies and Research:
I am submitting herewith a dissertation written by Mahmoud Ali Bishr entitled "Development of a Mutant Strain of Escherichia coli for Molecular Cloning of Highly Methylated DNA." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

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Dedicated to my wife, Khadija, my children,
and
my parents, Ali Bishr & Zinab Khalil

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"In the name of Allah, Most Gracious, Most Merciful. Praise be to Allah. The Cherisher and Sustainer of the Worlds."

The Holy Quran 1:1

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Mahmoud Ali Bishr

DEVELOPMENT OF A MUTANT STRAIN OF
ESCHERICHIA COLI
FOR MOLECULAR CLONING OF HIGHLY METHYLATED DNA

August 1991

Supervisory professor: John E. Knesek, Ph. D.

A mutant strain of Escherichia coli designated as GR219 that allows efficient molecular cloning of highly methylated bean DNA has been developed by UV light mutation of the parent LE392 str^r strain.

This mutant strain, like the parent, is streptomycin resistant and is biologically contained, because it requires thymidine for growth. Both the wild type and the mutant strain have lambda phage receptors so both can be utilized for construction of genomic libraries using the phage as a vector.

The efficiency of transformation of the parent and the mutant strain with a recombinant plasmid containing bean DNA was compared to the efficiency of transformation of the PLK-F' strain, which has a deletion of *mcrA* and *mcrB* genes and, therefore, allows transformation with methylated bean DNA. It has been found that the GR219 strain has the highest efficiency of transformation, while the PLK-F' strain shows less, and the parent LE392 str^r strain the least efficiency of transformation.

These results indicate that strains of E. coli with *mcrA* and *mcrB* genes can recognize and degrade highly methylated DNA. However, other undefined factors affected by the altered gene(s) in the GR219 strain are also involved in the recognition and degradation of any cloned foreign DNA.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Amp	ampicillin
Amp^r	ampicillin resistant
BME	ß-mercaptoethanol
BSA	bovine serum albumin
CTAB	hexadecyltrimethylammonium bromide
DEAE	diethylaminoethyl
EDTA	disodium ethylenediaminetetraacetate
EtBr	ethidium bromide
NZY-T	NZY media plus thymidine
PAGE	polyacrylamide gel electrophoresis
PEG	polyethlene glycol
Sarkosyl	sodium sarcosinate
SDS	sodium dodecylsulfate
strept	streptomycin
str^r	streptomycin resistance
str^s	streptomycin sensitive
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
Tet	tetracycline
Tet^r	tetracycline resistant
Tris	Tris (hydroxymethylaminomethane)
UP H_2O	ultrapure water

CHAPTER I

INTRODUCTION

The isolation and characterization of a specific eukaryotic gene is very difficult because it constitutes such a small fraction of the total DNA. However, large quantities of a specific eukaryotic gene can be obtained by the use of the recombinant DNA technique which involves construction of novel DNA molecule by *in vitro* joining of DNA sequences from different sources. This technique was made possible by the discovery and isolation of the restriction endonuclease enzymes which can recognize certain specific base sequences leading to digestion of the DNA molecule producing small fragments with either blunt ends (no unpaired bases at the termini) or sticky ends (having a number of unpaired bases at either the 5'- or 3' termini) (Bolivar et al. 1979), then these fragments are ligated into a cloning vehicle like a bacterial phage or bacterial plasmid (known as a vector), and introducing this formed DNA into a host organism in which it can replicate (DNA transformation or transfection). As the recombinant phage or plasmid replicates its own DNA in a bacterial cell, the inserted DNA is also duplicated, thus yielding a large amount of this DNA.

The recombinant DNA techniques are useful for analyzing chromosome structure, because they allow chromosome segments bearing a specific gene to grow in quantities sufficient for study (Marx 1976).

One approach for isolation of a specific gene is the establishment of a genomic library. Libraries are constructed by combining fragments of total genomic DNA (passenger) with a vector under appropriate conditions resulting in recombinant molecules. The library is then screened or genetically selected for those cells that have replicated the desired recombinant molecule with a specific probe that can detect the clone containing the gene of interest (Morrow 1979). This technique is well established for animal cell DNA, but difficulties are often encountered, when cloning plant genomic DNAs. The reason why Escherichia coli does not accept plant DNA is that many strains of E. coli contain restriction modification systems that can recognize and degrade foreign DNAs.

These systems provide means whereby a bacterial cell can repel an invading bacteriophage or a plasmid transfer during conjugation. There appear to be two restriction modification systems for recognition and destruction of foreign DNAs. One system involves the restriction of modified DNAs like the mcrA and mcrB loci (modified cytosine restriction) and mrr (or mar) (methylated adenine recognition and restriction) that recognize the

modified base 5-methylcytosine (5-mC) and 6-methyladenine (6mA), respectively. The other system, which has restriction and modification activity, includes an endonuclease (ENase) which recognizes and degrades the unmodified bases and a methylase (MTase) which methylates the endogenous DNA, so the host cell can protect its own DNA (Raleigh 1987; Blumenthal 1989). The biological result of the restriction is degradation of the DNA and the molecular event, where it has been examined, is double strand cleavage. Since plant DNAs have high levels of these modified bases, the effects of mcr and mrr activities are especially relevant to cloning plant genomic DNAs.

The first report of restriction was published by Luria and Human (1952) and ironically dealt with the restriction of modified DNA, although this was not known at the time. This report involved E. coli mutants, subsequently shown to be defective for the production of UDP-glucose. Phages T2 or T6 could replicate once in these mutants but could not productively reinfect them. The phages liberated from such mutants were also unable to grow on wild type E. coli. This was later found to be due to the restriction of DNA containing 5-hydroxymethylcytosine (5-HmC) that has not been modified by addition of glucose. Phage containing cytosine instead of 5-HmC were not restricted. Two genetic loci rglA and rglB (restriction glucose-less phage) were found to be responsible for this restriction (Blumenthal 1989).

The phenomenon of restricting DNAs containing 5-mC was independently discovered by three groups during an attempt to clone bacterial and phage methylase genes (Blumenthal 1989; Noyer-Weidner et al. 1986; Raleigh and Wilson 1986). They showed that the genes formerly thought to restrict only DNAs containing 5-hydroxymethylcytosine present in non-glycosylated T-even phages can also recognize 5-mC. These products which restrict 5-mC containing DNA are encoded by *rglA* and *rglB* genes (restriction of glucoseless phage) (Woodcock et al. 1989).

Recently Raleigh and Wilson (1986) renamed these genes to *mcr* (modified cytosine restriction). However, the designations *rgl* and *mcr* can be considered interchangeable (Noyer-Wiedner et al. 1986; Raleigh and Wilson 1986).

Genetic mapping data and tests with the rgl mutants surmised that the *rgl* genes and the *mcr* genes are probably the same (Heitman and Model 1987; Raleigh et al. 1989). A third restriction system of this type has been identified by Heitman and Model (1987), which is active on adenine methylated DNA and is encoded by the mrr (mar) locus (methylated adenine recognition and restriction). These three restriction systems (Table 1) specific for methylated DNA are widespread among strains of E. coli and are designated as methylation-dependent restriction systems (MDRSs) (Blumenthal 1989).

Table 1: Escherichia coli loci that code for nucleases which are capable of recognizing and degrading cloned eukaryotic DNA.

Locus	Bases Recognized	Sequence
hsd	unmodified bases	AAC.N..N..NGTCG
mcrA	5-methylcytosine	C5mCGG
	5-hydroxymethylcytosine	G5mCGC
mcrB	5-methylcytosine	G5mCGC
	4-methylcytosine	AG5mCT
	5-hydroxymethylcytosine	GG5mCC
		CAGN4mCTG
mrr (mar)	6-methyladenine	GN6mATC
		CN6mAG
		CTGCN6mAG
		TCGN6mA-M

Very little is known about the structure and the biochemistry of the MDRS proteins. Evidence suggests that mcrA activity is membrane-associated and that mcrB activity is not. Both mcrB and mrr induce the E. coli recA dependent SOS response when the appropriate MTase gene is introduced, presumably due to DNA strand cleavage. Despite the brevity of its putative recognition sequence, the mcrB restriction of nonglucosylated 5-hydroxymethylcytosine containing phage DNA results in the accumulation of large fragments that are further degraded by RecBCD (exoV) activity (Blumenthal 1989).

All the MDR loci have been mapped and cloned. The mcrA locus is located on the 15 Kb, UV inducible excisable prophage like element e14 which is inserted at 24 min in K-12 strains of E. coli and contains several other interesting genes (Greener and Hill 1980; Raleigh et al. 1989; Bachmann 1983). The *mcrB* is located adjacent to the *hsdS* gene and the *mrr* is located adjacent to the *hsdR* gene. Both mcrB and mrr are located in a 14 Kb cluster at 98.5 min (Ross and Braymer 1987; Raleigh et al. 1989), that J. Hamilton nicknamed "The Ellis Island of E. coli," since these genes monitor the immigration of DNA (Blumenthal 1989). The mcrB locus has been sequenced and is composed of 2,695 base pairs, within the mcrB region of the chromosome in the *mcrB* gene, which encode both a protein of 51 Kilodaltons (KDs) (the *mcrB* gene product), and a protein of 39 KDs (the *mcrC* gene product) (Ross et

al. 1989). Two open reading frames were found in the sequence. They provide a specifying peptides of 455 and 348 amino acids which correspond to the products of the *mcrB* and *mcrC* gene, respectively. A single nucleotide overlap was found to exist between the termination codon of mcrB and the proposed inititation codon of *mcrC* (Ross et al. 1987; Rose et al. 1989). It is not clear if the two genes of the mcrB locus differ in their recognition of the different forms of the methylated cytosine. The mcrA phenotype is currently thought to be coded for by a single gene (*mcrA*) and there is some preliminary evidence that mrr involves two genes. The mcrA/rglA and mcrB/rglB identities have been confirmed through separate mapping of the respective mcr and rgl phenotypes in various mutants, and by complementation. It has been found that the rglA and mcrA phenotypes always coincide, as do the rglB and mcrB phenotypes (Raleigh et al. 1989).

The mcr and mrr nucleases influence the cloning of genomic DNAs in several ways. These enzymes seem to degrade methylated genomic DNA as it first enters the cell. However, any cloned DNA that escapes this initial degradation will replicate and lose its eukaryotic methylation pattern, thereby becoming insensitive to mcr activity. Therefore, the mcr systems do not absolutely preclude genomic cloning, but result in a significant reduction in the efficiency of the process (Raleigh 1987; Raleigh et al. 1988; Dila and Raleigh 1988; Whittaker et al. 1988; Woodcock et al.

1989). Recent evidence also suggests that the mcr nucleases can degrade genomic DNA during *in vitro* packaging reactions, resulting in a further decrease in cloning efficiency (Gossen and Vijg 1988; Kretz and Short 1989). Also mcr activity is probably a significant cause of bias in genomic libraries, resulting in both under-and over-representation of specific clones, depending on their particular methylation level (Woodcock et al. 1989; Graham et al. 1990).

Besides mcr activity, E. coli K12 has another restriction system which is capable of degrading cloned eukaryotic DNA. This restriction-modification function has been classified into three different categories according to their structural complexity and cofactor requirements (Yaun 1981; Yuan and Hamilton 1984). Enzymes of type I are heteropentameric, multifunctional proteins able to both cleave and methylate unmodified DNA. These enzymes are coded by genes in the bacterial chromosome, and are consistent with the presence of the genes in the restriction modification locus hsd (host specificity). There are 3 genes involved in restriction and modification, a restriction gene (hsdR), a modification gene (hsdM) and gene for recognition of host specificity site (hsdS) (Yaun 1981; Messelson et al. 1984; Loenen et al. 1987) (Table 1). Type II methylases, whose accompanying restriction enzymes are commonly used in recombinant DNA work, usually are encoded in plasmid genomes. These enzymes usually

are simple proteins, and require Mg^{2+} for activity. The DNA modification is catalyzed by separate methylase activity. Methylases of type III are similar to those of type I, but are heterodimeric multifunctional proteins and usually are encoded by bacteriophage P1 and plasmid P15 (Razin et al. 1984). All three types of restriction and modification systems require S-adenosyl-L-methionine (SAM) for methylation, while only types I and III require ATP and Mg^{2+} to stimulate activity (Yuan 1981). The hsd locus encodes the EcoK or EcoB restriction system. This enzyme system cleaves specific DNA sequences which are unprotected by methylation of N-6 specific adenine (Bickle 1982; Sain and Murray 1980).

Most plants, animals, and microorganisms contain a fraction (0.2-30%) of the cytosine or adenine residues in their DNA methylated in the 5'-position or 6'-position respectively. Among higher eukaryotes, DNA methylation is the rule. So far, the prodominant natural methylated base that has been found is 5-methylcytosine (Wyatt 1951; Vanyunish et al. 1970; Hall 1971). In plants, 40-50% of all cytosine may be methylated, including over 80% of the CG and $C^{A}/_{T}G$ sequences (Gruenbaum et al. 1981; Razin 1988). In higher plant DNA, the 5-methylated cytosine content is usually higher than in animal DNA (Hall 1971). Methylated DNA has been determined in different plants and the following values have been found (Wagner and Capesius 1981): Lobularia maritiuma 18.5%,

Nicotiana tobacum 34.6%, Pisum sativum 23.2%, Rhimanthus minor 29.2%, Sinapsis alba 12.2%, Vicia faba 30.5%, Visium album 23.2%, Cymbiium pumiliam 18.8%, Cymbidium pumilum 15.8%, Tritium aestivum 22.4%. The degree of methylation has been found to vary in different plant structures and during plant ontogeny. For example, determination of methylated cytosine of several plants by thin-layer chromatography revealed a higher degree of methylation in the DNA of generative organs than in the DNA of vegetative organs (Khvoyka et al. 1978). Furthermore, methylated cytosine in lettuce DNA was higher at 24 h than at 18 h after germination. These results were attributed to methylation lagging somewhat behind DNA replication and cell development.

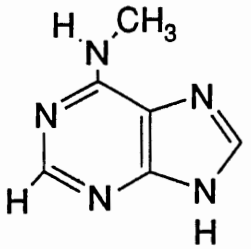
DNA methylation occurs as a postreplicative process on the nascent DNA daughter strand close to the growing fork and results in methylation of all available sites. Studies by Billen (1968) and Lark (1968) confirmed the model *in vivo* in E. coli by analyzing DNA in cesium chloride gradients after various treatments. If E. coli was starved of methionine, DNA synthesis continued, but such DNA was not methylated. On restoration of methionine, DNA methylation occurred before subsequent replication. In normal cells (not starved) DNA methylation was concomitant with synthesis, and thus, the two processes probably are coupled. Also studies by Bird (1978) and by Szyf et al. (1982) revealed that no hemimethylated DNA sequences could be found on DNA strands,

suggesting that methylation occurred at the replication fork and resulted in methylation of the cytosines on both strands.

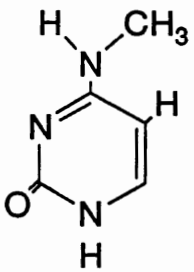
In addition to the nuclear DNA being methylated, organellar DNA (mitochondrial and chloroplast) is also methylated. The 5-mC content of these DNA's is usually much lower than that of nuclear DNA. However, in some plants the 5-mC content of mitochondrial and chloroplast DNA has been reported to be similar to or higher than that of the nuclear DNA (Ehrlich and Wang 1981).

In addition to the 5-mC and 6-mA there are some other modified bases that have been detected in different DNAs (Figure 1), such as 4-methylcytosine (4-mC), which is the product of DNA modification by the *Bcn* 1 methylase, and the *mom* gene of phage mu (Janulaitis et al. 1983). A modified cytosine has also been detected in the DNA of Trypanosomes species which is not 5-methylcytosine, but its chemical nature has not been elucidated (Adams and Burdon 1985). These methylated bases are usually a small fraction of the major nonmethylated bases, but the situation in the DNA of the phage Xp-12 and the T-even bacteriophages are quite distinct. In these phage DNAs, all cytosines are completely modified to 5-hydroxymethylcytosine (5-HmC) which in turn can be glycosylated (Kuo et al. 1968; Ehrlich et al. 1975; Adams and Burdon 1985). Similarly, in the DNA of various Bacillus subtilis phages, the thymine can be completely replaced with

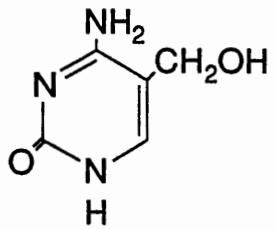
5-Methylcytosine
(5mC)



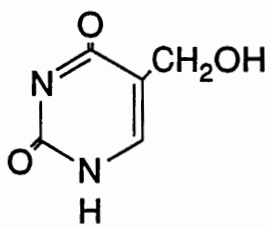
6-Methyladenine
(6 mA)



4-Methylcytosine
(4mC)



5-Hydroxymethylcytosine
(5HmC)



5-Hydroxymethyluracil

Figure 1 : The Minor Bases Found in DNA

hydroxymethyl uracil (Kallen et al. 1962; Takahashi and Murmer 1963).

DNA methylation involves an enzyme which catalyze transfer of a methyl group from S-adenosyl-L-methionine (SAM) (Appendix A) to either the 5-position on the cytosine ring or the 6-amino group of adenine (Appendix B), but unlike most eukaryotic DNA the 5-methylcytosine residues of the XP-12 phage derive their methyl groups from the 3-carbon of serine instead of the thiomethyl carbon of methionine, in a reaction involving tetrahydrofolic acid (Ehrich and Malanie 1975; Adams and Burdon 1985). Eukaryotic DNA methyl transferases, have no cofactor requirement and are not stimulated by ATP or Mg^{2+}. Surprisingly there is, as yet, no report describing the isolation and characterization of a plant DNA methyl transferase. However, it is believed that the principal characteristic of plant DNA methyl transferase will turn out to be similar to the known features of the mammalian enzyme that has been characterized and described. Nevertheless, it should be kept in mind that mammalian methylase modifies exclusively CpG sequences, while the plant methylase must recognize and methylate CpXpG as well (Razin 1988). So, in plant cells, it is possible that two methyl transferases exist. One would be similar to the animal enzyme, methylating cytosine in CpG dinucleotides and a second would be acting on CpNpG trinucleotides (Razin et al. 1984).

Beside the restriction function, DNA methylation plays several other roles. It has been suggested that the essential function of the 5-methylcytosine is to modify protein-DNA interactions. The conversion of cytosine to 5-mC introduces a methyl group into an exposed position in the major groove of the DNA helix, and the binding to the DNA of proteins such as lac repressor, histones, and hormone receptors is known to be affected by changes in the major groove (Razin and Riggs 1980). It has been found that there are some proteins that can differentiate between methylated and unmethylated sequences and these proteins are more likely to be important in mediating some effects of eukaryotic DNA methylation on chromosome function (Zhang et al. 1989). More recently a 5-methylcytosine specific DNA binding protein has been isolated from plant cells (DBPm). This protein can recognize the 5-methylcytosine residues without appreciable DNA sequence specificity unlike the mammalian DNA-binding proteins (MDBP) which recognize 5-methylcytosine residues in a related family of 14 base pair sequences (Zhang et al. 1989). The complexity of the secondary and tertiary folding of eukaryotic chromosomes which probably involves specific protein-DNA interactions which could be influenced by the distribution of 5-mC in the DNA and the high methylation of centromeric regions suggests a possible role of DNA methylation in mitosis and chromosome sorting and packing.

DNA methylation plays a role in DNA replication in some phages, since methylation at one specific site is known to be involved in replication and blocking methylation blocks DNA synthesis. The replication of the E. coli chromosome also stops after one round of synthesis in the absence of methylation. Under normal growth conditions the replication origin sequence of E. coli (GATC) is ten times more highly methylated than similar sequence in the genome. Together these observations suggest a possible role for DNA methylation in prokaryotic DNA replication (Razin and Riggs 1980). However, analytical studies by Szyf et al. (1982) on the methylation pattern of specific sequences, such as the origin of replication and rRNA genes using wild type and methylation deficient (dam- dcm-) mutant strains of E. coli, showed that these sequences were methylated in wild type cells and unmethylated in mutant cells. Since these mutant cells grew normally, the origin of replication and the *rRNA* genes must function normally, regardless of whether these sequences are methylated or not. An attractive possibility that receives increasing support is a role of methylation in the transcriptional regulation of DNA and, thereby, in cell differentiation (Scarano et al. 1965; Vanyushin 1974). The 5-methylcytosine appear to stabilize portions of the DNA and this stabilization might be efficient to help regulate DNA replication as well as transcription, because both processes are believed to involve local denaturation of the DNA (Bick et al. 1972). The 5-

methylcytosine plays a role in vertebrate gene replication and possibly in embryonic development. However, Drosophila melanogaster does not have any detectable methylated bases (Urieli-shovals et al. 1982), and manages to undergo complex development without DNA methylation. Therefore, methylation may not be essential as the only fundamental level of control of gene expression during development (Razin et al. 1984).

In recombination, mutation and oncogenesis, recent results obtained with prokaryotes suggest that the role of DNA methylation in mutation should be considered seriously. Methylase-deficient E. coli mutants have been isolated that have reduced methylation of GATC sequences. These mutants show increased spontaneous mutagenesis, increased spontaneous induction of lambda prophage, increased sensitivity to mutagens and ultraviolet light, and hyperrecombination. It has been suggested after analysis of nearest neighbor dinuceotide frequencies that mutation (deamination) of 5-methylcytosine generates thymine which is not preferentially corrected and leads to a heritable change in the DNA (GC to AT transition) that could influence differentiation. In contrast, deamination of cytosine produces uracil, which is recognized and preferentially corrected. Although this is an interesting possibility, there has been little experimental support for this idea. Recent evidence showed that some thermophilic bacteria have 4-methylcytosine in their DNA's, instead of the 5-

methylcytosine as the minor base. These modified bases allow the bacteria to be more resistant to heat induced deamination and conversion to thymine residues (Ehrlich et al. 1985).

It has been known for some time that the doublet CpG is rare in eukaryotic DNA. Salser et al. (1978) have pointed out that if methylated CpG sites were hot spots for mutations, evolution would tend to eliminate them except where there was positive selection. This model suggests that noncoding regions will show a scarcity of CpG sequences relative to coding sequences. Considerable interest has been shown in the methylation of tumor cell DNA and the total content of 5-mC during carcinogenesis, and it has been found that the methylation pattern changes during cell transformation, but that demethylation of specific cellular gene sequences may be an important event in the transformation process. More recently, it has been postulated that DNA methylation facilitates chromatin condensation and it may be essential for rapid cell division, a characteristic of tumor cells (Cooper 1983; Riggs and Jones 1983).

It has been proven that E. coli restriction systems have significant effects on reducing the efficiency of cloning of methylated DNAs and the ability to generate libraries representative of the gene population from these DNAs. This is true for libraries generated through both plasmid transformation and phage infection. The hsd system has the broadest inhibitory

activity, since it can act on unmodified DNA bases (Kretz and Short 1989), and so the removal of this inhibition activity is essential for efficient library construction. Inactivation of the mcrA, mcrB, mcrC, and mrr activities of E. coli have also been demonstrated to cause a higher yield of recombinant phage containing methylated DNA (Lorow and Jesse 1990). A wide variety of eukaryotic DNA is considerably methylated at cytosine residues, with methylation of plant DNA exceeding the level found in most mammalian DNAs. Thus, due to cellular systems which recognize, restrict, and degrade methylated residues, construction of representative libraries from such methylated DNA is difficult, unless all the restriction activities are inactivated or eliminated from the host strain or from both the plating and the lambda packaging extract.

In the construction of genomic libraries, it is advantageous to use phage as a vector, because larger DNA fragments can be inserted as compared to cloning in plasmids. In our laboratory, we observed that very few stable bean DNA recombinants are formed. One explanation for the lack of the recombinants is that the E. coli host strains destroy the recombinant vectors by endogenous nucleases that recognize and degrade the methylated bases of the bean DNA. Another possibility is that bean DNA is toxic to the E. coli cells and results in death of the bacteria. Therefore, research was initiated to see if a mutant strain of E. coli could be developed that will allow more efficient molecular cloning of bean DNA. Even

though methylated DNAs may be the major obstacle in cloning of the recombinant vectors containing plant DNAs using E. coli as a host, there may be other factors involved which reduce the ability to maintain the recombinant vectors. Therefore, the selection of a mutant in which to clone methylated bean DNA was not limited to mutation of the *mcr* genes which recognize and degrade methylated DNA in E. coli.

To select a mutant srain of E. coli which tolerates methylated DNA, a plasmid was used as a vector. Plasmid recombinants are easier to prepare and use for E. coli transformation and would also provide an easy selection of bacterial strains which will tolerate methylated DNA. The plasmid pNO1523 was used as a vector because it allows direct selection for recombinant DNA molecules unlike most vectors which are indirectly selected by antibiotic resistance. As well direct selection of pNO1523 is provided by cloning into the Sma I restriction site within the *rplS* (*strA*) gene.

In order to take advantage of this characteristic of the plasmid, E. coli LE392 was used as a suitable host, because: (*i*) it can be converted to competency; (*ii*) it is biologically contained on account of its thymidine growth requirement; (*iii*) it has receptors for lambda phage, allowing the use of this phage as a vector.

THE SPECIFIC AIMS:

To obtain the objective of this research the following specific aims were carried out:

1) Develop a streptomycin resistant (str^r) mutant of strain E. coli LE392 to allow direct selection of recombinants.

2) Confirm the mutation in *rpsL* gene by transformation of the mutant E. coli cells with plasmid pNO1523 which carries the wild type gene, and cure the cells of the plasmid by growing the cells at higher temperature.

3) Mutate the E. coli LE392 str^r with UV light and select for a mutant that allows efficient cloning of bean DNA.

4) Compare the efficiency of the transformation of this mutant strain with a strain of E. coli that has deletions in the *mcrA* and *mcrB* genes.

5) Characterize the genetic make-up of this mutant strain for thymidine requirement, streptomycin resistance, and lambda phage receptors.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

Enzymes, reagents, and organisms: Phaseolus vulgaris seeds (cultivars: contender and commodor) were purchased from Harpool's Seed Company, Inc., Denton, Texas. Restriction endonucleases and T4 DNA ligase were obtained from Betheseda Research Laboratories, Gaithersburg, MD. Acrylamide-bisacrylamide (29:1) solution ampicillin, streptomycin, and tetracyclin were purchased from American Research Product Company, (AMRESCO) Salon, OH. Thymidine, chloramphenicol and hexadecyltrimethylammonium bromide (CTAB) were obtained from Sigma chemical company, Saint Loius, MO. Proteinase-K cellulose was obtained from EM Labs, Inc. New York, NY. NAP-5 columns were purchased from Pharmacia, Uppsala, Sweden. Other regeants were used in these experiments were obtained from commercial supplier and are of the best grade and quality avaliable.

The NZY powdered media was obtained from Gibco Laboratories Inc., Grand Island, NY, and the agar was obtained from

Difco Laboratories, Detriot, Michigan. The NZY broth media and agar plates were prepared according to Maniates et al. (1982). The NZY agar plates containing antibiotics were prepared by addition of 50 ug/ml ampicillin (amp), 30 ug/ml streptomycin (str), and 40 ug/ml of tetracycline (tet) after autoclaving and cooling of the media to about 56 ^{o}C. All the NZY agar plates containing streptomycin are also contained thymidine in quantity of 50 ug/ml (NZY-T).

Growth and Storage of the bacterial strains:

Escherichia coli LE392 which requires thymidine for growth were kindly provided by Dr. Savio L. C. Woo at Baylor College of Medicine, Houston, TX. The bacteria were maintained by streaking on NZY-T agar plates. The E. coli GR219 cells were maintained by streaking on NZY-T agar plates plus sterptomycin. The E. coli MC1009 cells containing the plasmid pNO1523 which was provided (courtesy of Dr. Dennis Dean) were maintained by streaking on NZY agar plates containing antibiotic ampicillin and the E. coli PLK- F' was maintained by streaking on NZY agar plates containing tetracycline (Table 2). Long term storage of the bacterial cells was done by mixing 100 ul of bacterial culture in NZY broth (A_{600nm} = 0.8) with 200 ul of sterile 80% glycerol in a 2 ml screw cap tube, followed by gentle agitation, and storage at -70 ^{o}C.

B. METHODS

DNA PREPARATION

Large scale plasmid DNA preparation: E. coli MC1009, was streaked on NZY agar plates containing ampicillin. After overnight incubation at 37 °C, one colony was selected and transfered into 1000 ml flask containing 250 ml NZY broth media and incubated at 37 °C with shaking (about 200 rpm) until the absorbance reach a value of A_{600nm} = 0.8, then 1 ml of a chloramphenicol solution (50 mg/ml dissolved in absolute alcohol) was added. The bacterial cells were incubated overnight at 37 °C with shaking, and then were harvested by centrifugation in 250 ml screw top bottle in a JA-14 rotor at 5K rpm for 15 min at 5 °C using a Beckman J-21B centrifuge. The bacterial pellet was resuspended in 5 ml of 20% sucrose contaning 0.05 M Tris-HCl, pH 8. The bacterial cells were lysed by adding 2 ml of freshly prepared lysozyme solution (10 mg/ml in ultrapure water) and gently mixed and set on ice for 10 min. Then 25 ml of 50 mM Tris-HCl, and 20 mM disodium ethylenediaminetetraacetate (EDTA), solution pH 8, 2.5 ug of proteinase K-cellulose and 1.5 ml of 10% sodium dodecylsulfate (SDS) were added followed by incubation at 56 °C in a water bath. The SDS solution was added just prior to the incubation. After one hour incubation, 38 ml of digested preparation 17.5 ml of 5 M NaCl, and 2 ml of 10% SDS were added

Table 2: Bacterial Strains and Plasmid

Plasmid or Organism	Source/Reference
Escherichia coli LE392 thy	Dr. Savio Woo, Houston ,TX
Escherichia coli MC1009	Dr. Dennis Dean, Madison ,WI
Escherichia coli PLK- F'	Stratagene, La Jolla, CA
Escherichia coli DH-5 alpha	BRL-Gaithersburg, MD.
Plasmid pN01523	Dr. Dennis Dean, Madison, WI

and the bottle was placed in the incubator at 37 ^{o}C. After overnight incubation, the bottle was placed at -20 ^{o}C for 2 h followed by centrifugation at 12K in a JA-14 rotor in a Beckman J-21B centrifuge for 20 min at 4 ^{o}C. The supernatant was transferred to another screw top bottle and nucleic acids were precipitated with the addition of two volumes of absolute ethanol and storage at -20 ^{o}C overnight. The sample was warmed to room temperature for about 1 to 2 h and the DNA was pelleted by centrifugation at 6K rpm at 20 ^{o}C in a JA-14 rotor for 20 min and the supernatant was discarded and 200 ml of 70% ethanol was added and allow to set at room temperature for 1 to 24 h followed by centifugation at 6K rpm at 20 ^{o}C in a JA-14 rotor for 20 min. The pellet was dissolved in 9 ml of ultrapure water and 1.0 ml of 0.5 M sodium citrate, pH 8.0 was added. After one hour incubation at 68 ^{o}C, 9.43 gm of cesium chloride (CsCl), and 400 ul of ethidium bromide (EtBr) (10 mg/ml) were added to 10 ml of the DNA solution. The mixture was centrifuged in a Type 80 Ti rotor at 55,000 rpm for 17 h at 20 ^{o}C using Type 80 Ti quick seal polyallomer tube in a Beckman L8-70 centrifuge.

The lower plasmid band was collected with a 1.0 ml syring using 18 gauge needle and extracted 4 times with equal volume of isopropanol. The DNA was desalted by passing through a NAP-5 column. The column was equiliberated with a total volume of 10 ml of ultrapure water (UP H_2O), and then 0.5 ml of the DNA sample

was added to the column and then exactly 0.75 ml of UP H_2O was added to the column. The DNA sample was collected in 2.0 ml eppendorf tubes. The concentration of the DNA samples were determined spectrophotometrically by diluting 10 ul of the DNA sample to 300 ul with UP H_2O. The absorbance at 260 was used to calculate concentration of DNA as follows: the absorbance value at 260nm x 30 x 50 = microgram of DNA/ml.

Isolation of high molecular weight bean DNA:

Phaseolus vulgaris seeds (cultivars: contender and commodor) were planted to germinate in plastic pots containing Trophy potting soil. The pots were maintained in a growth chamber with a periodic watering for about six weeks. After germination, the leaves were harvested weighted, and washed in 0.5% hexadecyltrimethyl-ammonium bromide (CTAB) solution followed by a sterile water rinse. The rinsed leaves were either used immediatly or frozen in plastic bages at -70 ^{o}C until needed for DNA extraction. The DNA was isolated from the fresh or frozen leaf tissue using the CTAB method (Murray and Thomas 1980; Taylor and Powell 1982; Doyle et al. 1990). The leaf tissue was ground to a fine powder with liquid nitrogen in a sterile mortar, then ß-mercaptoethanol (BME) (2% v/w) was added followed by addition of an equal volume of freshly prepared boiled 2X extraction buffer (2% CTAB w/v, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl). The mixture was transferred to a water bath at 55 ^{o}C with a manual stirring until the mixture

temperature reached 50 °C. The tissue solution was gently extracted with an equal volume of chloroform: isoamyl alcohol (24 :1) and centrifuged for 10 min at 3.5K rpm at 20 °C in an IEC CU 5000 centrifuge with an 8 place head. The upper, aqueous layer of the mixture was collected and 1 ml of a 10% CTAB solution was added for each 10 ml of solution. The mixture was reextracted with chloroform: isoamyl alcohol and centrifuged as above and the upper, aqueous layers were combined and an equal volume of precipitation buffer [1% CTAB w/v, 50 mM Tris-HCl pH8.0, 10 mM EDTA, 1% BME (added just prior to use)] was added. The solution was gently mixed, incubated at room temperature (25 °C) for 30 min, and then centrifuged at 3K rpm for 5 min at 20 °C in an IEC CU 5000 centrifuge with an 8 place head. The pellet was dissolved in 9 ml of UP H_2O, 1.0 ml of 0.5 M sodium citrate and incubated in water bath for 1 h at 68 °C. After cooling to room temperature, 9.43 gm of CsCl and 400 ul of ethidium bromide (10 mg/ml) was added to the solution.

The mixture was transferred to a Beckman quick seal polyallomer tube and centrifuged in a Beckman Type 80 Ti rotor at 55,000 rpm for 17 h at 20 °C in Beckman L8-70 ultraspeed centrifuge. The DNA band was collected using 1.0 ml syring with an 18 gauge needle and the DNA was extracted four times with an equal volume of isopropanol. The cesium chloride was removed by

passing the DNA through NAP-5 column and the DNA concentration was determined spectrophotometrically.

Bacterial DNA preparation: The E. coli DH-5 alpha cells were streaked on NZY agar plates and grown overnight at 37 ^{o}C. A fresh colony was inculated into 250 ml flask containing 50 ml NZY broth and incubated at 37 ^{o}C while shaking until, the bacterial growth reached an absorbance value of A_{600}nm = 0.8. The bacteria were placed in 50 ml conical tube and centrifuged at 3400 rpm in a PRJ centrifuge at 0 ^{o}C. The bacterial pellet was resuspended in 2.5 ml of a solution containing 20% sucrose in a 0.05 M Tris-HCl pH 8.0. One milliliter of freshly prepared lysozyme (10 mg/ml of UP H_2O) was added. The mixture was placed on ice for 30 min, then 250 ul of 5% sarkosyl solution and 2.0 mg of proteinase K were added and the mixture was incubated in water bath at 56 ^{o}C for 1 h. The mixture was then diluted to 10 ml using solution containing 50 mM Tris-HCl and 20 mM EDTA, pH 8.0. Cesium chloride (9.43gm) and 400 ul of ethidium bromide (10 mg/ml) were added, and the mixture was centrifuged at 55K rpm at 20 ^{o}C for 17 h in a Type 80 Ti rotor in a Beckman ultraspeed centrifuge.

The bacterial DNA was collected using 1 ml syring with an 18 gauge needle. The DNA was extracted four times with equal volume of isopropanol, and the cesium chloride was removed by passing the DNA through NAP-5 column. DNA concentration was determined spectrophotometrically.

ANALYSIS OF DNA

Preparation of DNA for ligation reaction: The bacterial and bean DNA samples (20 ug) were double digested with 3 ul Alu I (30U) and 2 ul (20U) Hae III restriction endonuclease enzymes and buffers at 37 ^{o}C, and the plasmid DNA (10 ug) was digested using 1 ul (10U) of Sma 1 restriction enzyme and buffers (Appendix C) at 30 ^{o}C. All the restriction digests mixtures were incubated overnight. These three enzymes produce blunt end fragments. After digestion, the DNA samples were subjected to ethanol precipitation in a SW 41 polyallomer tubes by adding 0.2 volumes of 5 M NaCl and 3 volumes of absolute ethanol. The tubes were covered with parafilm and stored at -20 ^{o}C overnight or longer, then the tubes were filled with 70% ethanol and centrifuged at 39,000 rpm for 1.5 h in SW 41 rotor in Beckman L8-70 centrifuge at 20 ^{o}C. The pellets were then dried under vacuum overnight in a LabConco lypholizer LABCONCO, Kansas City, Mo. The DNA pellet was resuspended in UP H_2O, at a final concentration of 1 ug/ml. Ligation of DNA samples was carried out using T4 DNA ligase and ligation buffer [0.5 M Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM ATP, 500 ug of Bovine serum albumin (BSA) and 25% (w/v) Polyethylene glycol 8000 (PEG), where 250 ng of the plasmid DNA was ligated with 0.0001, 0.001, 0.01 and 0.1 ug of either bacterial or bean DNA (Appendix D). The ligation reactions were allowed to proceed overnight at room temperature. Ten

microliters of each of the ligated DNA was mixed with 200 ul of the competent bacterial cells and allowed to take the DNA on ice as described in the preparation of competent bacterial cells for transformation section.

Gel Electrophoresis: DNA was analyzed on either polyacrylamide or agarose gels. The polyacrylamide gels (5%) were prepared by mixing 38 ml UP H_2O, 6.4 ml of 40% acrylamide bis-acrylamide solution, 5 ml of 5X Tris-borate/EDTA buffer (TBA) (1X final conc. = 89 mM Tris, 89 mM boric acid, 2 mM EDTA), 500 ul of 10% ammonium persulfate and 50 ul of TEMED. The solution was pipetted into a vertical 14 X 16 cm Hoefer Model SE 400 gel unit. The gels were electrophoresd with 0.5X TBE buffer at 80 V for 2 to 3 h. After electrophoresis, the gels were stained with EtBr (1 ug/ml) for 30 min. Stained gels were photographed with Polaroid 57 film under UV illumination (Transilluminator Model TR-302 Spectroline).

Agarose gels (usually 0.8%) (Seakem GTG agarose FMC BioProducts, Rockland, ME), were prepared with 1X Tris acetate/EDTA (TAE) buffer (40 mM Tris-acetate pH 8.0, 2 mM EDTA) and 1X TAE was also employed as the electophoresis buffer. Ten micoliters of sample were mixed with 3 ul tracking dye (0.25% bromophenol blue, 20 mM EDTA pH 7.0, 50% glycerol) and were electrophoresed on the gels at 40 V for 2 to 3 h on the Hoefer Scientific Instuments Model HE 33 unit. After electrophoresis, the

gels were stained with EtBr (1 ug/ml) for 10 to 30 min and photographed (Polariod 57 film) under UV illumination at 302 nm (Transilluminator Model TR-302 Spectroline).

Preparation of competant bacterial cells: E. coli cells were rendered competent by standared transformation procedures (Mandel 1970). One colony was picked from NZY-T agar plates, inocubated into 50 ml NZY-T broth and incubated at 37 ^{o}C with shaking until the culture reached an absorbance value of A_{600}= 0.6. The cells were chilled and pelleted by centrifugation at 0 ^{o}C for 5 min at 3400 rpm in an IEC PRJ centrifuge with a 12 place head. The cell pellet was resuspended in 50 ml cold buffer A (25 mM Tris-HCl, pH 7.5, and 10 mM NaCl). The cells were centrifuged again for 5 min at 3400 rpm and the pellet was resuspended in 25 ml of cold buffer B (25 mM Tris-HCl, pH 7.5, 10 mM NaCl and 50 mM CaCl). The cell suspention was chilled on ice for 30 min, then centrifuged at 0 ^{o}C for 5 min at 1800 rpm and the pellet was gently resuspended in 5 ml of buffer B. DNA for transformation (10 ul) was added to 200 ul of the cell suspension and was chilled on ice for 30 min followed by heat shock for 1 min at 40 ^{o}C, then the suspension was placed again on ice for 10 min. Fifty microliters of this mixture were spread on appriopate agar plates. The plates were sat at room temperature for 30 min then incubated in the incubator at 37 ^{o}C overnight.

MANIPULATION OF BACTERIAL CELLS

Mutation of S12 ribosomal protein of E. coli LE392 thy: Fifty microliter of E. coli LE392 bacterial culture in NZY broth at A_{600nm} = 0.8, were exposed to ultraviolet light for 15, 30, and 60 sec at a distance of 25 cm from a General Electric germicidal lamp 2200-2600 A (220nm-260nm). The objective of this treatment is to cause mutation in the *rpsL* (*strA*) gene which is responsible for the production of the ribosomal protein S12 involved in cellular sensitivity to the antibiotic streptomycin. This mutation needs to be verified by streaking some individual colonies on NZY-T agar plates containing streptomycin. The mutated E. coli LE392 str^r cells were made competent and transformed with the plasmid pNO1523 which contain the normal *rpsL* gene. Bacterial cells were then streaked on NZY-T agar plates contaning ampicillin (NZY-T amp) and NZY-T agar plates containing ampicillin and streptomycin (NZY-T amp-str).

Mutation of the bacterial cells for efficient transformation with bean DNA: The E. coli LE392 str^r cells was allowed to grow in 250 ml flask containing 50 ml NZY broth to A_{600nm} = 0.6, then 100 ul of the bacterial growth suspension was spread into NZY-T agar plates containing streptomycin. The plates were exposed to ultraviolet light treatment using a Stratalinker (Stratagene, La Jolla, CA) at energy 15,000-20,000 joules. The mutation experiments were designed to result in about 5% survival

of cells, so ten fold serial dilutions of the bacterial suspension were made by mixing of 900 ul of UP H_2O or 0.9 % saline solution with 100 ul of successive bacteria suspension, then 100 ul of each dilution was spread into NZY-T agar plates containing streptomycin. All the plates were incubated at 37 ^oC overnight in the incubator. Each colony that grew on the exposed plates was picked and transferred into 250 ml flasks containing 50 ml NZY-T broth incubated in a shaker at 37 ^oC until the A_{600nm} = 0.6 was reached. Cells were rendered competent by the standared procedure, and then the bean-plasmid DNA ligation mixture was added to the competent cells. Fifty microliters of the bacteria suspension were spread on NZY-T amp-strept agar plates incubated overnight at 37 ^oC. Bacterial colonies from plates with a high efficiency of transformation were picked and cured of the recombinant plasmid. These isolates were transferred to 125 ml flasks containing 25 ml of NZY-T broth, incubated in a shaker at 37 ^oC until the A_{600nm} = 0.6 was reached. These were made competent, and transformed with the plasmid-bacterial DNA, or with plasmid-bean DNA ligation mixture in order to compare the efficiency of the transformation using bean DNA verses bacterial DNA.

Elimination of the plasmid from bacterial cells: The E. coli LE392 str^r cells that have been transformed with the intact plasmid pNO1523 were subjected to a curing process to free them

from the plasmid. A single colony was picked from NZY-T amp agar plates and inculated into 250 ml flask containing 50 ml of NZY broth incubated at 45 ^{o}C-50 ^{o}C with shaking overnight. Then a loopful of the bacterial growth suspension was streaked into a NZY-T strept agar plates and incubated at 37 ^{o}C overnight. Individual colonies were streaked onto both NZY-T plates containing amp and NZY-T amp-strept plates and incubated overnight at 37 ^{o}C. Bacterial isolates that did not grow on these plates were free of the plasmid and are said to be cured.

The genetic make-up of the E. coli GR219 cells: The E. coli GR219 cells had a high efficiency of transformation using the plasmid-bean DNA. These cells were characterized in regard to the thymidine requirement, streptomycin resistance, and lambda phage receptors.

To test whether the E. coli GR219 cells still require thymidine for growth, the E. coli GR219 and LE392 str^{r} cells, were streaked on NZY-T, and NZY agar plates. The plates were incubated overnight at 37 ^{o}C in the incubator and examined for the presence or absence of growth.

To determine if the E. coli GR219 is steptomycin resistant, the bacteria were spread on NZY-T agar or Muller Hinton agar plates plus thymidine (MH-T). Streptomycin discs (10 mg) were placed on each plate. The plates were incubated overnight at 37 ^{o}C

in the incubator and examined for the zone of inhibition around the streptomycin disc.

To test for lambda phage receptors, the E. coli LE392 str^r and GR219 cells were streaked on NZY-T plus strerptomycin agar plates, and the E. coli PLK-F' strain was streaked on NZY- tet agar plates. After overnight incubation at 37 ^oC, colonies from each strain were inculated into a 250 ml flask containing 50 ml NZY broth with 1% maltose and were incubated in a shaker incubator until the absorbance value A_{600nm} = 0.6 was reached. Five metal capped glass tubes (13 x 100mm), each containing 1 ml of 1.6% agarose and 1 ml of 4X NZY broth were then placed in a 56 ^oC heater block (Multi-block heater Lab Line Instruments, Inc., Melrose Park, Illinios) until ready for use. To five, 10 ml plastic tubes, 1 ml of SM buffer (2.9 g NaCl, 0.5 g $MgSO_4$, 2.5 ml Tris-HCl, pH 7.5, and 2.5 ml of 2% gelatin in H_2O) were added. Ten microliter of phage stock suspension (10^8 PFU/ml) were added and a hundred-fold serial dilutions were made giving a range of dilution of 10^{-2} through 10^{-10} dilutions. These dilutions were assayed by adding 1 ml of appropriate strain to each dilution tube and combining them with the melted agarose NZY broth mixture. The contents of each tube were mixed by inverting the tube twice and the content were poured into the selected NZY agar plates. The plates were swirled to insure complete coverage of the surface, the lids were wiped with Kimwipes and the plates were incubated

in the incubator at 37 ^{o}C (not inverted). After overnight incubation, the plates were examined for presence or absence of the plaques.

CHAPTER III

RESULTS

The purpose of this research was to develop a mutant strain of E. coli which will allow molecular cloning of highly methylated DNA by altering the host restriction systems that recognize and degrade highly methylated DNA. The plasmid pNO1523, was used as a cloning vector that allowed direct selection of plasmid containing foreign DNA. However, to use this plasmid requires a strain of E. coli with a mutated S12 protein that will not bind the antibiotic streptomycin.

The E. coli strain LE392 thy, was selected as a suitable host for this study because it can be made competent for high efficiency of transformation and it has a receptor for lambda phage so it could be used for genomic library construction with the phage. Furthermore, since this strain requires thymidine for growth, it is biologically contained.

To induce mutation, E.coli LE392 cells were exposed to ultraviolet light from a General Electric germicidal lamp with the intent of mutating the *rpsL* (*str A*) gene. To select for streptomycin resistance, the bacterial colonies that grew on NZY-T

plates after the exposure to the ultraviolet light were picked and streaked on NZY-T str agar plates. Thus, E. coli LE392 strain was used to select for streptomycin resistance in order to use pNO1523. As well, these colonies may also have mutations in the *rpsL* (*strA*) gene. To prove that the E.coli LE392 *rplS* (*strA*) gene had been mutated, some colonies that grew on NZY-T plus streptomycin were transformed with the plasmid pNO1523 which contained a wild *rpsL* (*strA*) gene and amp resistant gene. The transformed cells were seeded on NZY-T amp and NZY-T amp-strept agar plates and incubated overnight. The colonies on each plate were counted. Bacterial colonies were observed on NZY-T amp plates and no colonies were detected on the NZY-T amp-strept agar plates (Table 3). These results indicated that the cells had a mutated *rpsL* (*strA*) gene, since the wild *rps* L gene, present in pNO1523 plasmid, made the mutated bacterial cells resistant to ampicillin and sensitive to streptomycin (Table 3).

To obtain the mutated host cell free of the plasmid, one of the isolates, designated as E. coli LE392 str^r, was cured of the plasmid by growing at high temperature. It was then streaked on NZY-T amp, NZY-T str, and NZY-T amp-str agar plates, and incubated overnight. The bacteria grew on NZY-T str but not on the other two media, indicating that the bacteria lost the plasmid, thus rendering the cells to be streptomycin resistant and ampicillin sensitive. Having the E. coli LE392 str^r and the direct

Table 3: Transformation of E. coli LE392 str^r with the plasmid pNO1523.

	Number of Colonies	
Volume[a]	NZY-T Amp	NZY-T Amp Strep
10 ul	135	No growth
20 ul	300	No growth
25 ul	300	No growth
50 ul	TMTC[b]	No growth
75 ul	TMTC	No growth
100 ul	TMTC	No growth

[a] Volume of the transformed bacterial cells plated on 100 mm plates.
[b] Too many to count.

selection vector pNO1523, one can answer the question whether plasmid vectors containing bean DNA transform E. coli less efficiently than plasmid vectors containing bacterial DNA. To answer this question the following experiments were performed.

The DNA recombinant molecules were constructed by digesting the plasmid DNA with Sma I enzyme, and the bacterial and bean DNA were digested with Alu I and Hae III enzymes in order to obtain small DNA fragments from both bacterial and bean DNA. The DNA fragment(s) produced by these digestions were blunt ends. The three DNA samples were subjected to agarose gel electrophoresis on 0.8% agarose. The plasmid DNA formed one band of 5.2 Kbp (Figure 2; Lane 5), and the bean and bacterial DNAs, formed a smear of DNA fragments which migrated faster than the 500 bp fragment of the Hind III cut lambda (Figure 2; Lane 1 and 3) respectivily. Different concentrations of the cleaved bacterial or bean DNAs were ligated with Sma I digested plasmid and the ligated mixture was added to competent E. coli LE392 str^r cells. These were plated on NZY-T amp-str agar plates. After overnight incubation, the colonies were counted. Higher numbers of colonies were observed with plasmid-bacterial DNA than with plasmid-bean DNA (Table 4). These results indicated that the transformation of E. coli LE392 str^r was more efficient using bacterial DNA than bean DNA. The percent of reduction of transformation using bean DNA has been found to range between 61% to 74.5% (Table 6).

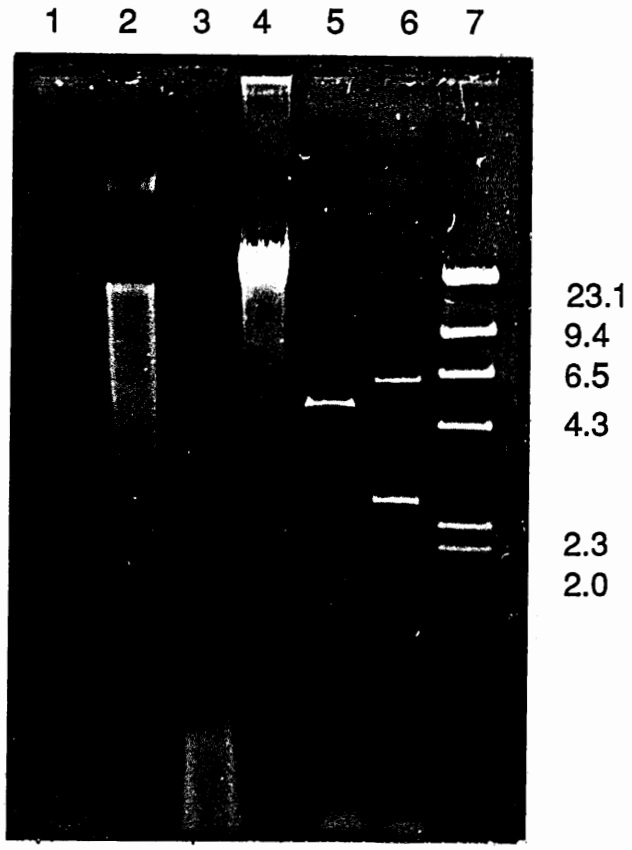


Figure 2. Analysis of restriction endonuclease digested plasmid, bacterial, and bean DNA by agarose gel electrophoresis using 0.8% agarose in 1X TAE buffer at 40 V for 3 h. Lane 1, bean DNA Alu I and Hae III cut; Lane 2, bean DNA uncut; Lane 3, bacterial DNA Alu I and Hae III cut; Lane 4, bacterial DNA uncut; Lane 5, plasmid pNO1523 Sma I cut; Lane 6, plasmid pNO1523 uncut; Lane 7, lambda Hind III cut marker.

Table 4: Number of the transformants obtained from transformation of E. coli LE392 str^r by plasmid containing either bacterial or bean DNA.

Concentration of DNA in the reaction mixture (ug/ml[a])	Experiment Number 1		2		3	
	Bact	Bean	Bact	Bean	Bact	Bean
0.01	651	189	819	210	1008	252
0.1	798	252	966	252	1134	357
1.0	1134	336	1428	462	1344	567
10.0	1953	756	1932	735	1953	798
100.0	3360	1344	4158	1428	3612	1176

[a] Concentration of bacterial or bean DNA added to 250 ng of Sma 1 digested pNO1523 in the ligation mixture.

Do E. coli cells recognize and degrade bean DNA because it is highly methylated? To answer this question, bacterial and bean DNA were cleaved with Cla I enzyme, and the samples were subjected to agarose gel electrophoresis. The bacterial DNA sample revealed smears of small DNA fragments (Figure 3; Lane 1), but no smear was revealed with the bean DNA (Figure 3; Lane 3). These results indicate that the bacterial DNA has been digested into small size fragments, but the bean DNA migrated as one band slightly larger than the 23 Kbp of lambda Hind III cut marker, suggesting that little or no digestion of the DNA occured.

To obtain a strain of E. coli LE392 str^r that would be more efficient in cloning of highly methylated bean DNA, E. coli LE392 str^r was exposed to ultraviolet light to cause about 95% lethality. The surviving colonies were picked, and each was grown and made competent. These were transformed using 0.0001, 0.001, 0.01, 0.1 and 1 ug of bean DNA ligated to 250 ng of Sma I digested plasmid pNO1523. Fifty microliters of the transformed cells were spread on NZY-T amp-str agar plates. The plates were incubated overnight at 37 ^{o}C and the colonies were counted after 24 h incubation. A colony from the isolate that gave the highest number of transformants was selected, cured, and designated as the GR219 strain.

E. coli GR219 was tested to determine the efficiency of transformation with plasmid-bean DNA and plasmid-bacterial DNA.

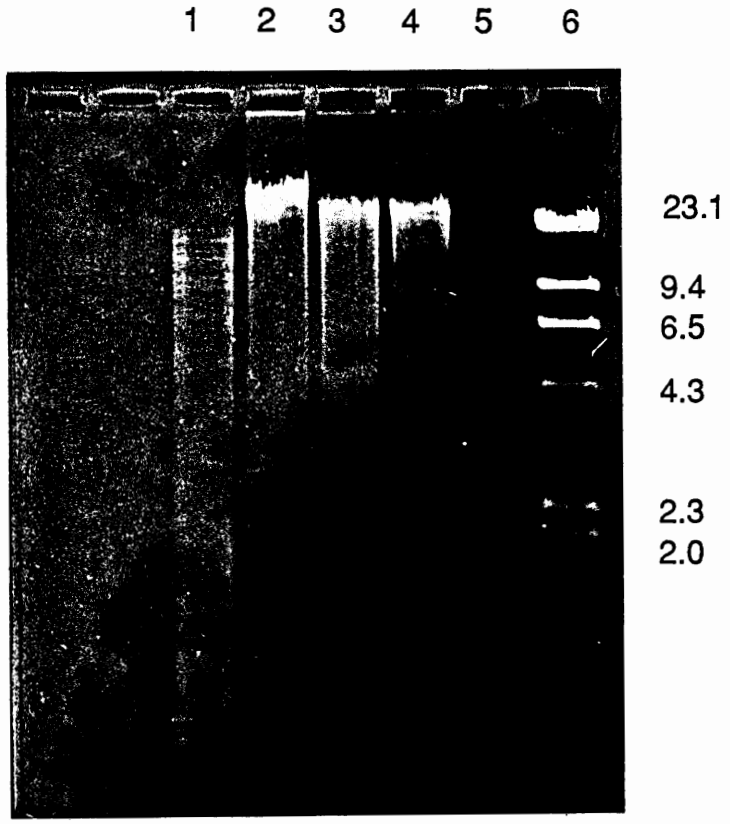


Figure 3. Analysis of restriction endonuclease digested bacteria and bean DNA by agarose gel electrophoresis using 0.8% agarose in 1X TAE buffer at 40 V for 3 h. Lane 1, bacterial DNA Cla I cut; Lane 2, bacterial DNA uncut; Lane 3, bean DNA Cla I cut; Lane 4, bean DNA uncut; Lane 5, blank; Lane 6, lambda Hind III cut marker.

The results showed that the total colony counts for the two recombinant DNA molecules were very similar (Table 5). The efficiencies of the transformation of the E. coli LE392 str^r and the E. coli GR219 cells were compared. The results showed that the new strain E. coli GR219 was more efficiently transformed with the bean DNA than was the parent strain E. coli LE392 str^r (Table 6).

The E. coli strain PLK-F' tet^r, which has deletions in the *mcrA* and *mcrB* genes, was also used as host for the recombinant plasmid-bacteria DNA and plasmid-bean DNA. Fifty microliters of the transformed cells were spread on NZY amp-tet agar plates and the colonies were counted after 24-48 h incubation. The results showed that by adding DNA to 100 ug/ml in the ligation mixture, there were more colonies from plasmid-bacterial DNA than from plasmid-bean DNA (Table 7).

Comparison of the three strains of E. coli with respect to their relative efficiencies of transformation using bacterial or bean DNA containing plasmid, revealed reductions in colony counts of 64.5, 19.1, and 7.5 for LE392 str^r, PLK-F', and GR219 respectively (Table 8 and Figure 4). Thus, it is concluded that GR219 strain is more premissive for maintaining methylated DNA, than either the LE392 or PLK-F' strain.

To assure that E. coli GR219 cells maintained their desirable characteristics, streptomycin sensitivity, thymidine requirement,

Table 5: Number of the transformants obtained after transformation of E. coli GR219 by plasmid containing either bacterial or bean DNA.

Concentration of DNA in the reaction mixture (ug/ml[a])	Experiment Number 1		2		3	
	Bact	Bean	Bact	Bean	Bact	Bean
0.01	1176	924	1197	987	1134	966
0.1	1512	1344	1554	1407	1785	1533
1.0	1743	1575	1869	638	2142	1827
10.0	2121	1932	2499	2205	2415	2247
100.0	3948	3738	3927	3528	4032	3759

[a] Concentration of bacterial or bean DNA added to 250 ng of Sma 1 digested pNO1523 in the ligation mixture.

Table 6: Comparison of the transformation efficiency of E. coli LE392 str^r and E. coli GR219 by plasmid containing either bacterial or bean DNA.

Concentration of DNA in the ligation mixture (ug/ml[a])	Percent reduction of transformation with plasmid containing bean DNA[b].	
	E. coli LE392 str^r	E. coli GR219.
0.01	74	18
0.1	70.5	11.7
1.0	65.5	12
10.0	61.0	9.3
100.0	64.5	7.5

[a] Concentration of bean DNA added to 250 ng of Sma 1 digested plasmid pNO1523 in the ligation mixture.

[b] percent reduction of transformation = 100% - (number of colonies with bean DNA/number of colonies with bacterial DNA x 100). (An average of three experiments).

Table 7: Number of transformants obtained after transformation of E. coli PKL-F' by plasmid containing either bacterial or bean DNA.

Concentration of DNA in reaction mixture (ug/ml)[a]	Exp. 1		Exp. 2	
	Bact	Bean	Bact	Bean
100.0	3528	2835	5229	4263

[a] Concentration of bacterial or bean DNA added to 250 ng of Sma1 digested plasmid pNO1523 in the ligation mixture.

Table 8: Comparison of the three strains of E. coli with respect to the reduction of transformation with plasmid containing bean DNA[a]

Host Strain	Ratio between Bacteria and Bean DNA[b]	Percent reduction of transformation with plasmid containing bean DNA[c]
E. coli LE392 str^r	2.80	64.5
E. coli GR219	1.07	7.5
E. coli PKL-F'	1.20	19.1

[a] These data were obtained using 100 ug/ml of bacterial or bean DNA added to 250 ng of Sma1 cut plasmid pNO1523.

[b] No. of transformants obtained with bacterial DNA relative to transformants obtained with bean DNA.

[c] The percent of transformation reduction has been calculated using the following formula : 100 % - (number of colonies with bean DNA/number of colonies with bacterial DNA x 100).

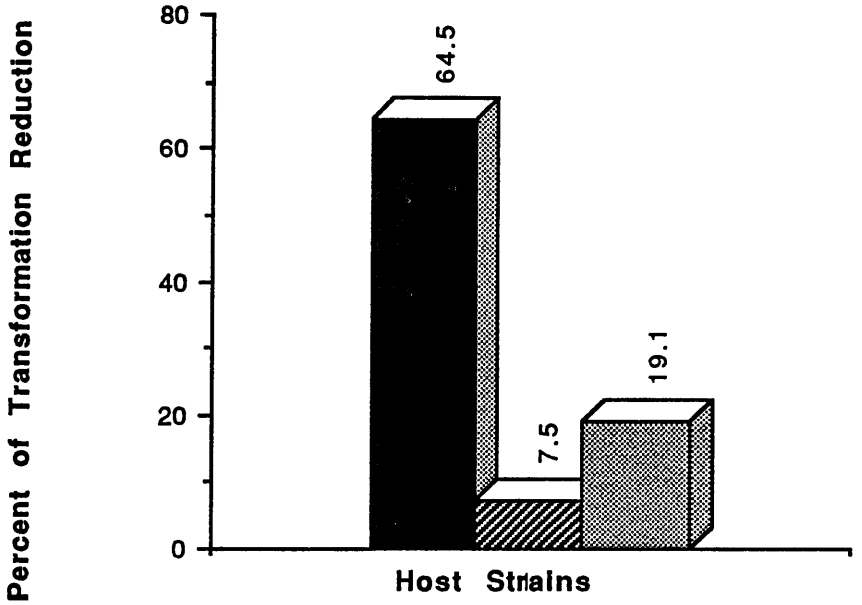


Figure 4. Comparison of the percent of transformation reduction between E. coli strains LE392 str^r, GR219, and PLK-F'.
E. coli LE392 str^r; E. coli GR219; E. coli PLK-F'

and the presence of lambda phage receptors were evaluated. E. coli LE392 str^r and GR219 cells were streaked on NZY and NZY-T agar plates, and after overnight incubation the plates were examined for the presence or absence of growth. The results of this experiment demonstrated that neither of these strains could grow on the NZY (Figure 5). Thus, both LE392 str^r and GR219 strains still require thymidine for growth, and therefore are biologically contained.

These two strains were seeded on Mueller Hinton plus thymidine agar plates (MH-T) for streptomycin susceptibility testing. The results showed that both of the E. coli LE392 str^r and GR219 were resistant to streptomycin by showing no zone of inhibition around the streptomycin disc, compared to the sensitive strain LE392 (Figure 6; Plates A, B, and C). So, both strains are still streptomycin resistant and could be used for direct selection.

To determine the susceptibility of the bacterial strains to lambda phage infection, phage assay experiments were performed using E. coli LE392 str^r and GR219, as well as the PLK-F' as a positive control. Viral plaques were observed on the appropriate NZY agar plates using all three strains, indicating that these strains still possessed receptors for the lambda phage, and could be utilized for genomic library construction using the phage as a vector (Figure 7).

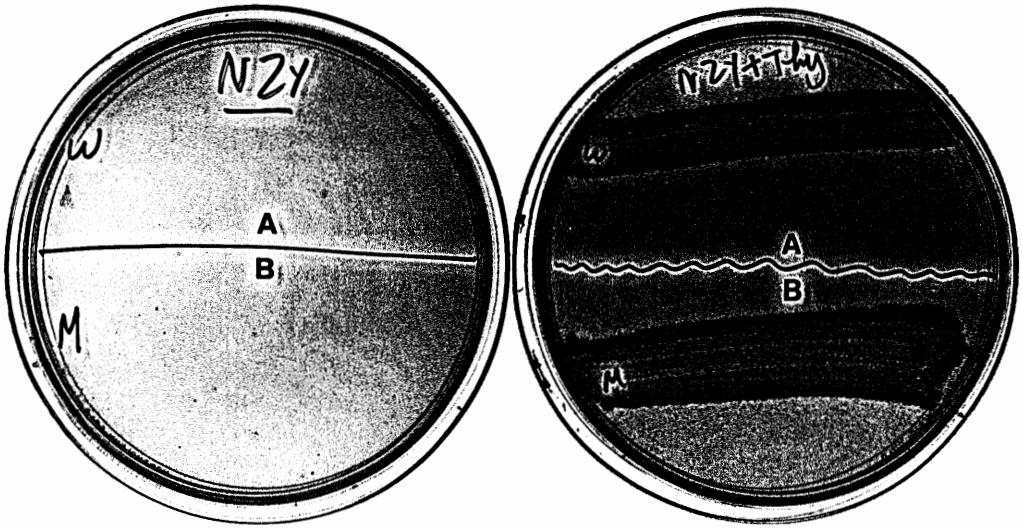


Figure 5: Comparison of growth of E. coli LE392 str^r and E. coli GR219 on NZY and NZY-T agar plates. A = E. coli LE392 str^r; B = E. coli GR219.

Figure 6: Comparison the susceptibility of the E. coli LE392 str^r and E. coli GR219 to streptomycin. Plate A = E. coli LE392; Plate B = E. coli LE392 str^r; Plate C = E. coli GR219.

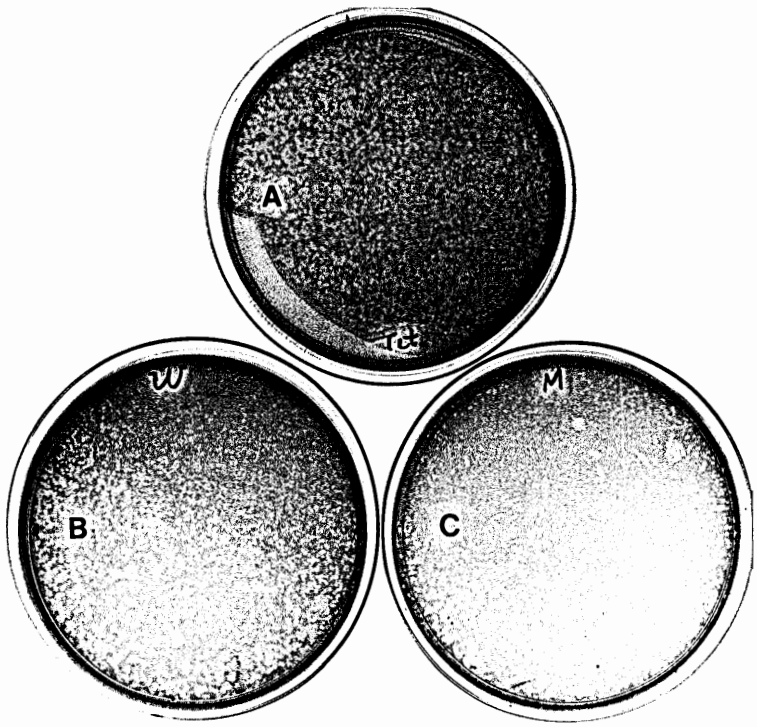


Figure 7: Susceptibility of E. coli LE392 str^r, E. coli GR219, and E. coli PLK-F' strains to infection with lambda phage. Plate A = E. coli PLK-F' strain; Plate B = E. coli LE392 str^r strain; Plate C = E. coli GR219 strain.

CHAPTER IV

DISCUSSION

In order to accomplish the objective of this research, recombinant DNA molecules needed to be constructed, and a system of selection needed to be devised. The essential ingredients for construction of a recombinant DNA molecule are a DNA vehicle (plasmid or phage vector), a DNA molecule to be replicated (passenger), and a method of joining the passenger to the vehicle (Morrow 1979). In addition, to perform a recombinant DNA experiment a mean of screening or genetic selection for those cells that have replicated the desired recombinant molecule has to be developed to insure that the transformed bacterial colonies have received both plasmid and insert and not just plasmid. Therefore, a selection system should be employed such that only the recombinant DNA molecule will successfully transform the bacterial host cells. Another consideration for performing recombinant DNA experiments that must be kept in mind is that the sizes of DNA fragments being cloned should be similar for the sake of comparison of transformation efficiencies.

The vector pNO1523 has an ampicillin gene (coding for ß-lactamase) and the *rplS* (*strA*) gene which codes for S12 ribosomal protein, responsible for cellular sensitivity to the antibiotic streptomycin. Sma I and Hpa I restriction sites are located within the *strA* gene. Therefore, when DNA fragments are cloned into the Sma I or Hpa I site and a streptomycin resistant strain is used as a recipient for transformation, the transformants will be resistant to both ampicillin and streptomycin. If the plasmid only is introduced into a streptomycin resistant strain, then that will cause the loss of the streptomycin resistant phenotype, unless the expression of the *rplS* gene carried on the plasmid has been inactivated. Thus, strains carrying recombinant plasmids can be directly selected in a single step by using a streptomycin resistant strain as host (Dean 1981; Post and Nomura 1980; Dean et al. 1981).

To take advantage of this direct selection system, E. coli LE392 was selected as a suitable host, because it could be efficiently converted to competency, it has receptors for lambda phage, and it is biologically contained on account of its thymidine growth requirement. E. coli LE392 was treated by exposure to ultraviolet light to cause mutation in the *rplS* gene and render the cells streptomycin resistant. This mutation needed to be verified by transformation of the cells with pNO1523 which carries the wild type *rplS* gene and amp resistant gene. The results as shown

in Table 3 indicated that the cells had lost streptomycin resistance and had acquired ampicillin resistance, due to this transformation confirming alteration of the *rplS* gene in the host cell.

To regain the mutant strain of E. coli in order to test its efficiency of transformation with bean DNA it was necessary that pNO1523 be removed. To accomplish this, a rescue curing experiment was performed by incubating the cells overnight at high temperature and a loopful was streaked on NZY-T str and NZY-T amp agar plates. Bacterial colonies were observed only on NZY-T str plates, implying that the bacterial cells were free of the plasmid and the cells were cured.

The loss of the plasmid from the bacterial cells could be explained by the fact that the plasmid DNA can not replicate at high temperature, possibly because some proteins required for plasmid DNA replication are inactivated at high temperature, or, as the bacterial cells replicate, the amount of plasmid DNA in comparison to the bacterial DNA is reduced resulting in loss of plasmid during cell segregation. This higher temperature treatment has less affect on the DNA replication of the E. coli host cells, because these bacteria are mesophilic with the ability to grow at temperatures between 20 ^{o}C and 45 ^{o}C. The heat-shock response proteins may play a role in maintaining and protecting the bacterial DNA, but not the plasmid DNA.

To verify that the recombinant vector with plant DNA is less effective in transforming E. coli than recombinant vectors with homologous bacterial DNA, bacterial and bean DNA each were cloned into the plasmid pNO1523. These recombinant molecules were used to transform the E. coli strain LE392 str^r.

To prepare for cloning experiments the DNA samples were exposed to restriction enzyme digestion where the bacterial and bean DNA each were separatly double digested with Alu I and Hae III enzymes while the plasmid DNA was digested with Sma I enzymes. All of these enzymes produce blunt ends. The DNA digestion was confirmed by running the samples on 0.8% agarose gel electrophoresis. The bacterial and bean DNAs digestion resulted in DNA that migrated faster than the 500 bp band of the lambda Hind III cut marker, and the Sma I digested plasmid DNA revealed one band at 5.2 Kbp (Figure 2). The digested DNA samples were ethanol precipitated, and then subjected to ligation reaction to construct the recombinant DNA molecules of plasmid bacterial and plasmid bean DNA (Appendix D). Each ligation mixture was used to transform the host cells E. coli LE392 str^r. The results indicated that the number of transformants produced by the bacterial DNA was higher than that of the bean DNA (Table 4). It was concluded that the bean DNA was cloned less efficiently than bacterial DNA, presumeably because bean DNA could not survive in the E. coli LE392 str^r host cells due to its recognition as foreign

DNA and its destruction by host restriction system(s) which recognize and degrade highly methylated DNAs (Ross et al. 1987). This leads to the conclusion that the bean DNA is highly methylated similar to other plant DNAs (Razin 1988; Blumenthal 1989).

To develop a mutant strain of E. coli that will allow efficient molecular cloning of highly methylated bean DNA, E. coli LE392 str^r was subjected to UV light and the surviving colonies were rendered competent and transformed with the recombinant molecule containing plasmid bean DNA. The resulting transformants were selected individually, cured, and retransformed with the same plasmid-bean DNA. The transformants were counted and compared with the number obtained with the plasmid-bacterial DNA. The transformation experiment was repeated with each colony until one was selected that gave a number of transformants closer to that obtained with the bacterial DNA. This strain was designated as GR219.

E. coli GR219 was transformed either with the same plasmid-bacterial DNA or with the plasmid-bean DNA, and the transformants from each were counted. The number of transformants from bean DNA was close to the number of the transformants from bacterial DNA (Table 5). The percent of the reduction of transformation was found to be higher with E. coli LE392 str^r than with GR219 (Table 6). These results indicated that E. coli GR219 no longer recognize

the bean DNA as foreign, probably due to alteration of the restriction system genes.

Do E. coli cells recognize bean DNA as foreign, because the bean DNA is highly methylated, or because some other factors are involved in the recognition of the bean DNA?

Bean DNA appears to be as highly methylated as other plant DNA. This hypothesis is supported by digesting bean and bacterial DNAs with Cla I an enzyme that is unable to cleave those methylated DNA sequences for which it is specific. The results yielded a smear of DNA fragments from the bacterial DNA, but showed that there was only one bean DNA band which migrated more slowly than the 23 Kbp lambda Hind III marker indicating the possibility that this DNA is not digested by Cla I due to the methylation of its recognition sequence (Figure 3).

To prove what exactly is involved in recognition and degradation of the highly methylated bean DNA, the E. coli strain PLK-F' tet^r in which the systems for restriction and degradation of methylated bases (*mcrA* and *mcrB* genes) were deleted, was used as a recipient for the recombinant DNA molecules. The number of the transformants and the percent reduction of transformation were calculated and compared among the three strains. It was found that the GR219 strain had the lowest percent of reduction of transformation (Table 8 and Figure 4). Therefore, it is clear that effects other than those involving the *mcrA* and *mcrB* genes are

responsible for recognition and degradation of the bean DNA. These could include *mrr*, which recognizes methylated adenine bases, or the *hsd* genes which selectively restrict DNA that is not protected by adenine methylation at the N-6 position in the sequence $A^{6me}A...N...GTGC$ or $GC^{6me}A...N...GTT$. Nucleases encoded by the *hsd* gene can not degrade the bacterial host cell DNA, because the adenine bases are protected by its restriction modification system (Yuan 1981; Yuan and Hamilton 1984).

Some aspects of the genetic make-up of E. coli GR219 were studied and compared with the parent strain E. coli LE392 str^r. It has been found that GR219 is still streptomycin resistant, so this can be used as a marker for cloning studies. It still requires thymidine for growth, a character which makes this strain, along with the parent strain, subject to biological containment. Also, GR219 still has receptors for the lambda phage, which is important for its use in construction of lambda vector genomic libraries.

In this study, the efficiency of transformation using homologous bacterial DNA and a methylated bean DNA was compared. It is clear that the efficiency of transformation of E. coli LE392 str^r with the bacterial DNA is higher than that using the bean DNA. The bean DNA is easily recognized as foreign DNA and restricted by host cell restriction systems, such as mcrA, mcrB, mrr, and hsd. Bacterial DNA might carry modifications similar to

those of the host cells and, consequently, would be considered as self.

In summary, to clone highly methylated plant DNA fragments, probably many of the host restriction systems must be inactivated, especially *hsd* which can recognize and destroy the unmodified bases which are found in any DNA, *mcrA* and *mcrB* which can recognize and degrade the modified cytosine bases which are found in high quantity in plant DNA (Graham et al. 1990; Kretz and Short 1989), and *mrr*, which produces a nuclease that can recognize and degrade the modified adenine bases which are found in some eukaryotic DNAs and may be found in plant DNAs.

An E. coli strain GR219 has been developed that can no longer recognize bean DNA as a foreign DNA. It has been demonstrated that the efficiency of transformation with vectors containing highly methylated plant DNAs is improved. However, additional studies should be performed to further characterize this strain, and more cloning experiments using additional bacterial, animal, and plant DNAs need to be done, in order to decide whether DNA methylation is the only factor involved in recognition and destruction of the highly methylated genomic DNAs or there are some other factors involved.

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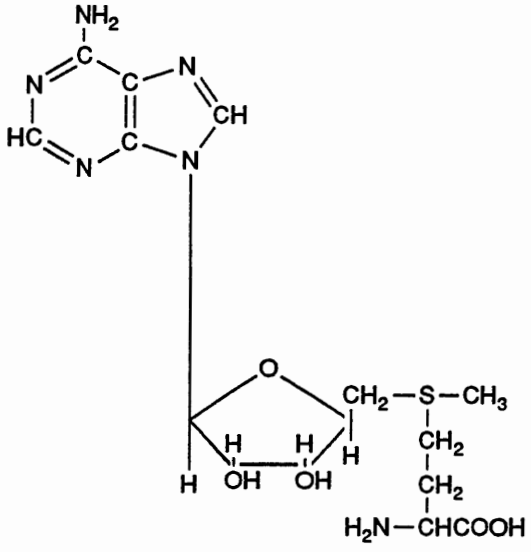
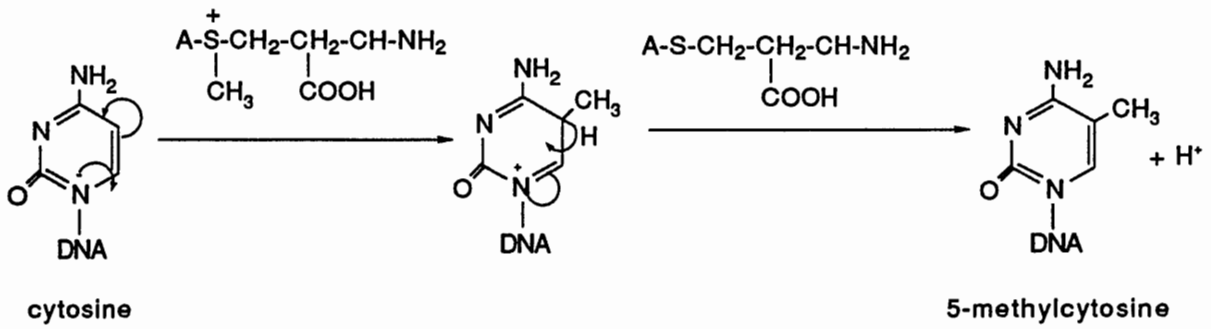
APPENDIX A



S-Adenosylmethionine

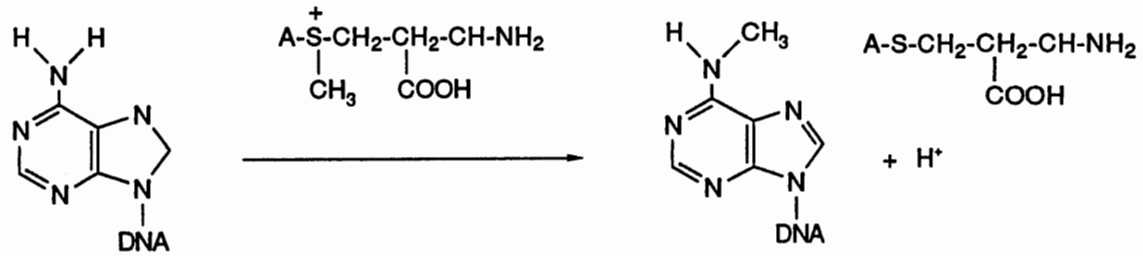
The methyl donor for transmethylations by DNA transmethylases

APPENDIX B



cytosine

5-methylcytosine



Adenine

6-methyadenine

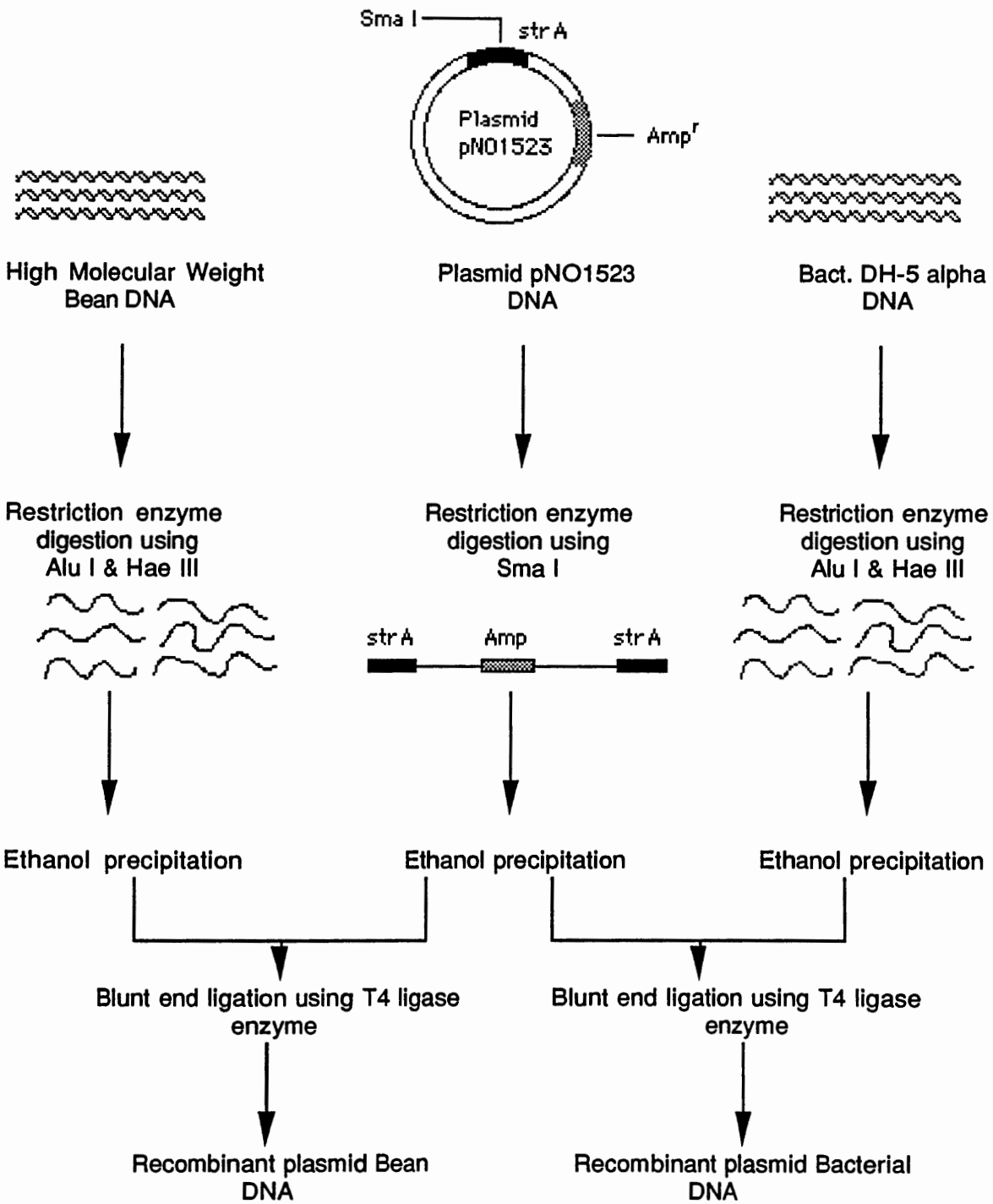
Synthesis of the minor methylated basis found in DNA

APPENDIX C

BRL 10X React Buffers 1X Final Concentration		Enzyme (U/ul s.a.)
React 1 -	50 mM Tris-HCl pH 8.0 10 mM MgCl2	Alu I (10 U/ul) Cla I (6 U/ul)
React 2 -	50 mM Tris-HCl pH 8.0 10 mM MgCl2 50 mM NaCl	Ava I (5 u/ul) Hae III (10 U/ul)
React 4 -	20 mM Tris-HCl pH 7.4	Sma I (10 U/ul) 5 mM MgCl2 50 mM KCl

BRL-Restriction endonuclease specific activity and buffer components.

APPENDIX D



Schematic diagram illustrating the construction of the recombinant DNAs.