IDENTIFYING FUNCTIONAL ROLES FOR ALKB IN THE ADAPTIVE RESPONSE OF *ESCHERICHIA COLI* TO ALKYLATION DAMAGE

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In loving memory of my Papa ji....

ABSTRACT

In 1977 a novel, inducible and error free DNA repair system in *Escherichia coli* came to light. It protected *E. coli* against the mutagenic and cytotoxic effects of alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS), and was termed the 'adaptive response of *E. coli* to alkylation damage'. This response consists of four inducible genes; *ada*, *aidB*, *alkA* and *alkB*. The *ada* gene product encodes an 0^6-methylguanine- DNA methyltransferase, and is also the positive regulator of the response. The *alkA* gene product encodes a 3-methyladenine- DNA glycosylase, and *aidB* shows homology to several mammalian acyl coenzyme A dehydrogenases. The *alkB* gene forms an operon with the *ada* gene, and encodes a 24 kDa protein. AlkB has been conserved from bacteria to humans indicating its importance, and although first described in 1983 its precise functional role still remains unknown.

This Ph.D. project describes advancements made in determining functional roles for the enigmatic *alkB* gene product. AlkB mutants are known to be highly sensitive to MMS. We report here that AlkB is not involved in repair of DNA strand breaks *in vivo*, or in repair of the major toxic lesion 3-methyladenine known to block DNA replication. A major step forward using bacteriophage host cell reactivation analyses has shown that AlkB is defective in repair of damaged single- stranded DNA. A thousand- fold defect was seen compared to a two- fold defect with damaged double stranded DNA. This result was specific to S_N2 alkylating agents, and its activity found to be independent of other known DNA repair pathways. AlkB mutants on exposure to MMS showed a low mutation frequency, and *in vitro* studies using various DNA substrates ruled out methyltransferase, glycosylase or any lesion transferral activities. Purified AlkB protein was shown to bind to DNA, with a greater affinity towards methylated single stranded DNA. Finally, we observed a greater sensitivity of *E. coli alkB* cells to MMS in an exponential phase of growth compared to cells in stationary phase. Thus we propose that AlkB is likely to act at single- stranded DNA regions within cells, such as at active sites of transcription and at replication forks.

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ABBREVIATIONS

AP	Apurinic/ Apyrimidinic
BCNU	N,N'-bis(2-chloroethyl)-N-nitrosourea
BER	Base excision repair
CPM	Counts per minute
DMS	Dimethyl sulfate
DNA	Deoxyribonucleic acid (SS- single stranded; DS- double stranded)
E. coli	*Escherichia coli*
γ- rays	Gamma rays
HPLC	High pressure liquid chromatography
λ- phage	Lambda bacteriophage
LB	Luria- Bertani medium

Lesions

1MeA	1-methyladenine
3MeA	3-methyladenine
7MeA	7-methyladenine
O^2MeC	O^2-methylcytosine
N^3MeC	N^3-methylcytosine
3MeG	3-methylguanine
O^6MeG	O^6-methylguanine
N^7MeG	N^7-methylguanine
O^2MeT	O^2-methylthymine
N^3MeT	N^3-methylthymine
O^4MeT	O^4-methylthymine
p(Me)	methyl phosphotriester

MeI	Methyl iodide

$MeOSO_2(CH_2)_2$-Lex	[1-methyl-4-[1-methyl-4-(3-(methoxysulfonyl)propanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido] propane. A methyl sulfonate ester attached to a non cationic minor groove binding N-methylpyrrole dipeptide (Lex). Designed mainly to produce N3-methyladenine damage.
MeOH	Methanol
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
mM	Milli molar
NER	Nucleotide excision repair
PAA	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
Plasmid	CC- Closed circular form OC- Open circular form
SCE	Sister chromatid exchange
SCX	Strong cation exchange
S_N	Nucleophilic substitution (S_N1 or S_N2)
UV	Ultraviolet light

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Chapter 1

CHAPTER 1

Introduction

Preface
Alkylation Damage
The Adaptive Response Of E. coli To Alkylation Damage
History Of AlkB
Overview Of Other DNA Damage And Repair
Aim

1.1 PREFACE

DNA repair has been described in the literature since the 1950's, but it was in 1994 that the 'DNA repair enzyme' was recognised as the molecule of the year (Koshland, 1994). This one molecule represents a whole range of enzymes involved in the many repair pathways existing to maintain the integrity of DNA.

Deoxyribonucleic acid (DNA) is the blueprint of life for every cell from bacteria to *Homo sapiens*, and before cell division must be copied with precision to allow survival. Failure to do so can lead to an increased frequency of cell death and disease. DNA is continually being damaged by both endogenous and exogenous agents. Therefore, cells have evolved many different repair systems to cope with these insults received to their genome. These processes manage to achieve a fine balance of fidelity, allowing only slight changes to occur in the genome to help maintain the evolutionary process. Overall the DNA repair pathways have been conserved from bacteria to man.

All of the DNA repair pathways known today are being studied in great detail.

New and exciting information is gained every year. Initial investigations often use simple model organisms such as *Escherichia coli* (*E. coli*) bacteria to understand the repair processes. Major advances in molecular biology techniques have allowed pathways to be investigated further in eukaryotes, and knocking out specific genes in mouse model systems may now allow us to understand their relevance towards disease.

This Ph.D. project focuses on a particular DNA repair pathway called the 'adaptive response of *E. coli* to alkylation damage'. Since its discovery the function of one gene product called *alkB* remains to be elucidated. Thus, initially described will be the adaptive response of *E. coli* cells, and the alkylation damage generated in DNA by different alkylating agents. AlkB was first described in 1983, and the knowledge gained over the years as regards this enigmatic protein will be discussed. Following this, although a large subject area an over-view of the other DNA repair pathways known, and the kinds of damage cells are exposed too is presented. This thesis then focuses on the progress that has been made towards identifying functional roles for AlkB. To conclude, the results obtained are discussed to provide a useful insight towards understanding the function of AlkB.

1.2 ALKYLATION DAMAGE

Alkylating agents are electrophilic compounds that have an affinity for nucleophilic centres i.e. negatively charged organic molecules such as DNA. Major sites of DNA methylation are shown in Figure 1. All alkylating agents react with DNA, and different sites in DNA have different reactivities. Generally ring nitrogens of the DNA bases are more nucleophilic than the oxygens, but both sites are subject to DNA methylation damage (Friedberg *et al.*, 1995). Alkylating compounds can be divided into monofunctional and bifunctional agents. Monofunctional compounds methylate DNA bases.

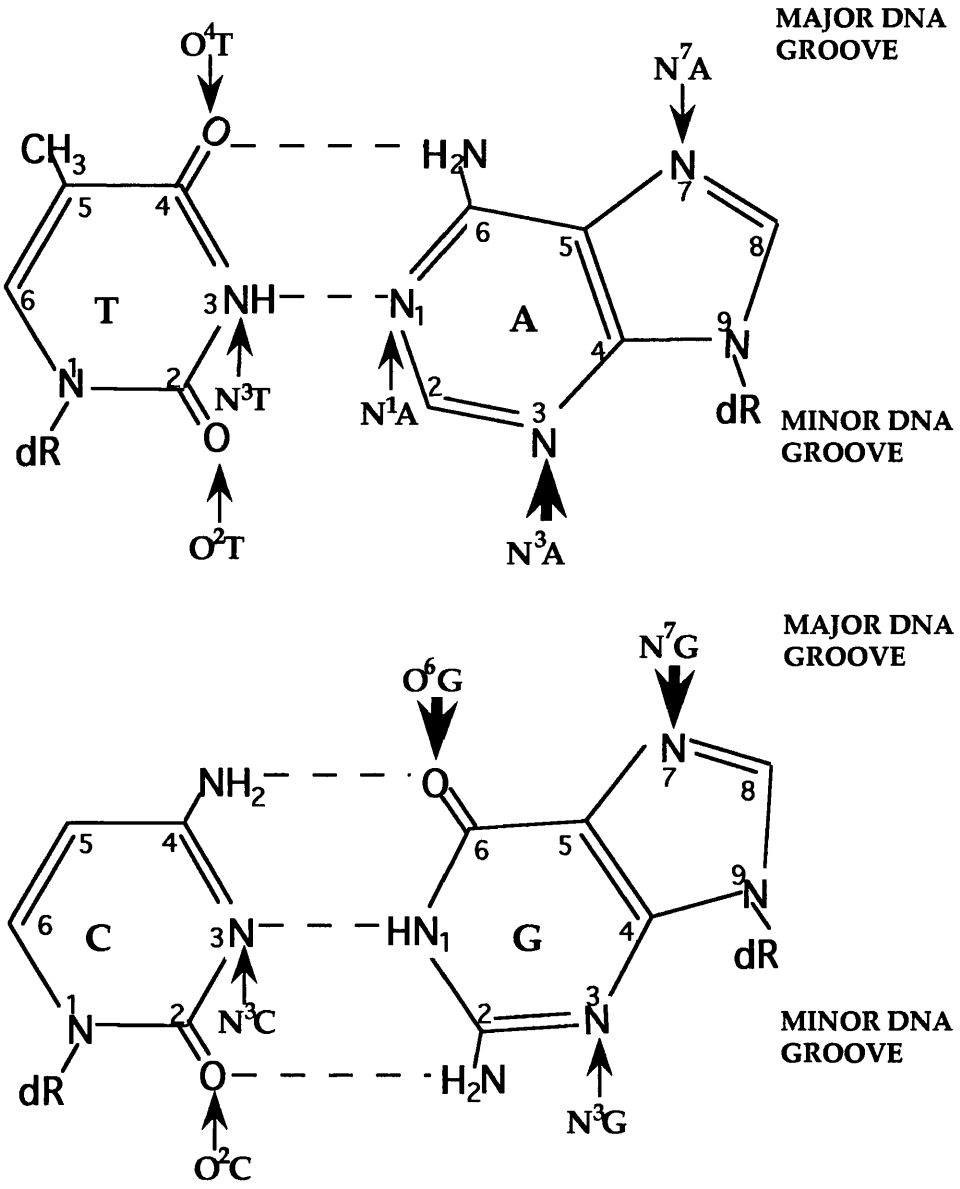


FIGURE 1: SITES OF DNA METHYLATION

Methylation of DNA occurs at several sites on all four DNA bases, although all sites do not show equal reactivity. **T**: Thymine; **A**: Adenine; **C**: Cytosine; **G**: Guanine. ➡ : Major sites of base damage; → Minor sites of base damage.

Bifunctional compounds e.g. N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU) do the same, but in addition can form cross- linkages or cyclise to form rings structures (Singer and Grunberger, 1983). Bifunctional agents exert cytotoxic affects at lower doses in comparison to their monofunctional analogues (Lawley, 1974).

Various alkylating agents exist environmentally and can methylate DNA. Bacterial systems have evolved the adaptive response to deal with alkylation damage, and this is discussed in the next section. Environmental alkylating agents arise from many sources and include methyl halides, certain antibiotics and the products of nitrosation reactions. The most abundant methyl halide is methyl chloride, a product of biomass burning or biological synthesis by algae and fungi. An antibiotic known to cause DNA damage is streptozotocin which produces the same methylated bases as MNU, as do nitrosation reactions such as that of methylurea with nitrite. Endogenously produced methylation damage is also known to occur from nitrosation reactions, and possibly from agents such as S-adenosylmethionine (minor contribution), produced when methionine reacts with adenosine triphosphate (Sedgwick and Vaughan, 1991; Taverna and Sedgwick, 1996; Vaughan *et al.*, 1991; Posnick and Samson, 1999).

Many different alkylating agents are used in the laboratory. Emphasis here is on monofunctional methylating agents, as they are the agents used for this study. Compounds are usually classified as the S_N1 or S_N2 group of alkylating agents, but can also be classified with respect to their Swain- Scott substrate factor (*s*) (Lawley, 1976). S_N1 and S_N2 refer to the mechanism of action based on the Ingold concept of nucleophilic substitution, (S_N). S_N1 reactions follow first order kinetics, and are dependent on formation of an intermediate carbocation. S_N2 reactions are bimolecular and dependent on steric accessibility of reacting species. S_N1 reactions are less selective for nucleophilic strength and can cause alkylation at hydroxyl groups, whereas S_N2 reactions by electrophilic agents

alkylate nucleophiles of high nucleophilic strength such as at nitrogen position seven of guanine, (N^7G) (Hemminki, 1983). The Swain- Scott factor refers to reactivity of agents compared to a standard of methyl bromide that is assigned *s* value of 1.0 and to water assigned zero. As one moves from the S_N1 to the S_N2 group of agents the Swain- Scott factor increases, and the ability of a compound to alkylate an oxygen atom site on a DNA base compared to a nitrogen site decreases, ($S_N1 \rightarrow S_N2$ O^6: N^7 alkylation $\downarrow$) (Beranek, 1990; Lawley, 1976). Examples of S_N1 alkylating agents include MNU and MNNG, and S_N2 agents MMS and DMS. The degree of DNA alkylation at different sites in DNA varies between the S_N1 and S_N2 compounds, and also is dependent on whether DNA is in its single or double stranded form. Using MMS and MNU as examples this variation is shown in Table 1, along with repair proteins associated with removal of different lesions.

Alkylated DNA bases in DNA can be potentially mutagenic and/ or cytotoxic to the cell. Cytotoxic lesions generally prevent hydrogen bonding between DNA bases and/ or block replication by DNA polymerases, so lead to inactivation of the DNA template. DNA base positions involved in hydrogen bonding are N-1 and N-6 of adenine; N-3 and O-4 of thymine; O-2, N-3, and N-4 of cytosine; and N-1, N-2 and O-6 of guanine. Reported lesions that are cytotoxic, and result from methylation of bases directly involved with hydrogen bonding include 1MeA, 3MeC, O^2T and 3MeT. Lesions 1MeA and 3MeC are not excessively produced in DS DNA due to steric hindrance and thus less accessibility of the alkylating agent to the site for DNA methylation, (see Table 1). Methylation of the DNA phosphate backbone to produce methyl phosphotriesters is also weakly cytotoxic, as the negative charges carried by these groups are lost. Promutagenic lesions in DNA include O^6MeG and O^4MeT. O^6MeG can mispair with thymine bases and O^4MeT with guanine bases. The result is production of G:C $\rightarrow$ A:T and A:T $\rightarrow$ G:C base transitions respectively. Overall most alkylation occurs at position N-7 of guanine, but

Modification	SINGLE STRANDED DNA % Of Total Alkylation		DOUBLE STRANDED DNA % Of Total Alkylation		Repair Protein
	MMS	MNU	MMS	MNU	
Adenine					
N1	18	2.8	3.8	1.3	
N3	1.4	2.6	10.4	9	AlkA
N7	3.8	1.8	(1.8)	1.7	
Guanine					
N3	~1	0.4	(0.6)	0.8	AlkA
O6	nd	3	(0.3)	6.3	Ada
N7	68	69	83	67	
Uracil/ Thymine					
O2				0.11	AlkA
N3				0.3	
O4				0.4	Ada
Cytosine					
O2			nd	0.1	AlkA
N3	10	2.3	(<1)	0.6	
Diester	2	~10	0.8	17	Ada

TABLE 1: NUCLEIC ACID BASE LESIONS INDUCED BY S_N1 (MNU) AND S_N2 (MMS) ALKYLATING AGENTS

Different lesions are generated upon alkylation of DNA by S_N1 (MNU) and S_N2 (MMS) agents. The degree of alkylation is different for single and double stranded DNA. nd: derivative not detected; (): estimated value, and so less reliable compared to other data shown. [Adapted from: Singer & Grunberger, 1983].

N^7MeG is an innocuous form of damage although it can lead to lethal depurinations (Lawley, 1974). 3MeA and 3MeG are also lethal lesions that block the minor groove of DNA, and thus block DNA replication (Karran *et al.*, 1982). Repair proteins are associated with most of these lesions, and as highlighted it is important to be able to remove them to ensure a low mutation frequency and cell survival.

1.3 THE ADAPTIVE RESPONSE OF *E. COLI* TO ALKYLATION DAMAGE

The adaptive response deals with damage incurred by alkylating agents. L. Samson and J. Cairns first described this pathway in 1977. Unexpected results were observed when *E. coli* cells treated with MNNG showed an increased resistance to the compound over time. There was no increase in mutation frequency, and so evidence pointed to induction of a novel repair pathway that was relatively error free (Samson and Cairns, 1977). Existing to specifically deal with alkylation damage, the adaptive response of *E. coli* is now characterised and shown to consist of four genes, *ada*, *alkA*, *aidB* and *alkB*, (Figure 2) (Lindahl *et al.*, 1988).

The 39kDa *ada* gene cloned in 1983 (Sedgwick, 1983) encodes DNA methyltransferase activity. It primarily works on double stranded DNA, and is the positive regulator of the adaptive response (Lindahl *et al.*, 1982). Ada contains two active domains joined by a central region of about ten amino acids. Active cysteine residues are contained within the C- terminal domain at position Cys-321 and the N- terminal domain at position Cys-69 (Demple *et al.*, 1985; Sedgwick *et al.*, 1988). Methyl groups from lesions O^6MeG and O^4MeT are transferred to Cys-321 resulting in a suicidal inactivation of Ada, and regenerating guanine and thymine in DNA. Methyl groups from methyl phosphotriesters (P(Me)s) are transferred to Cys-69 of Ada. Only the *S* –

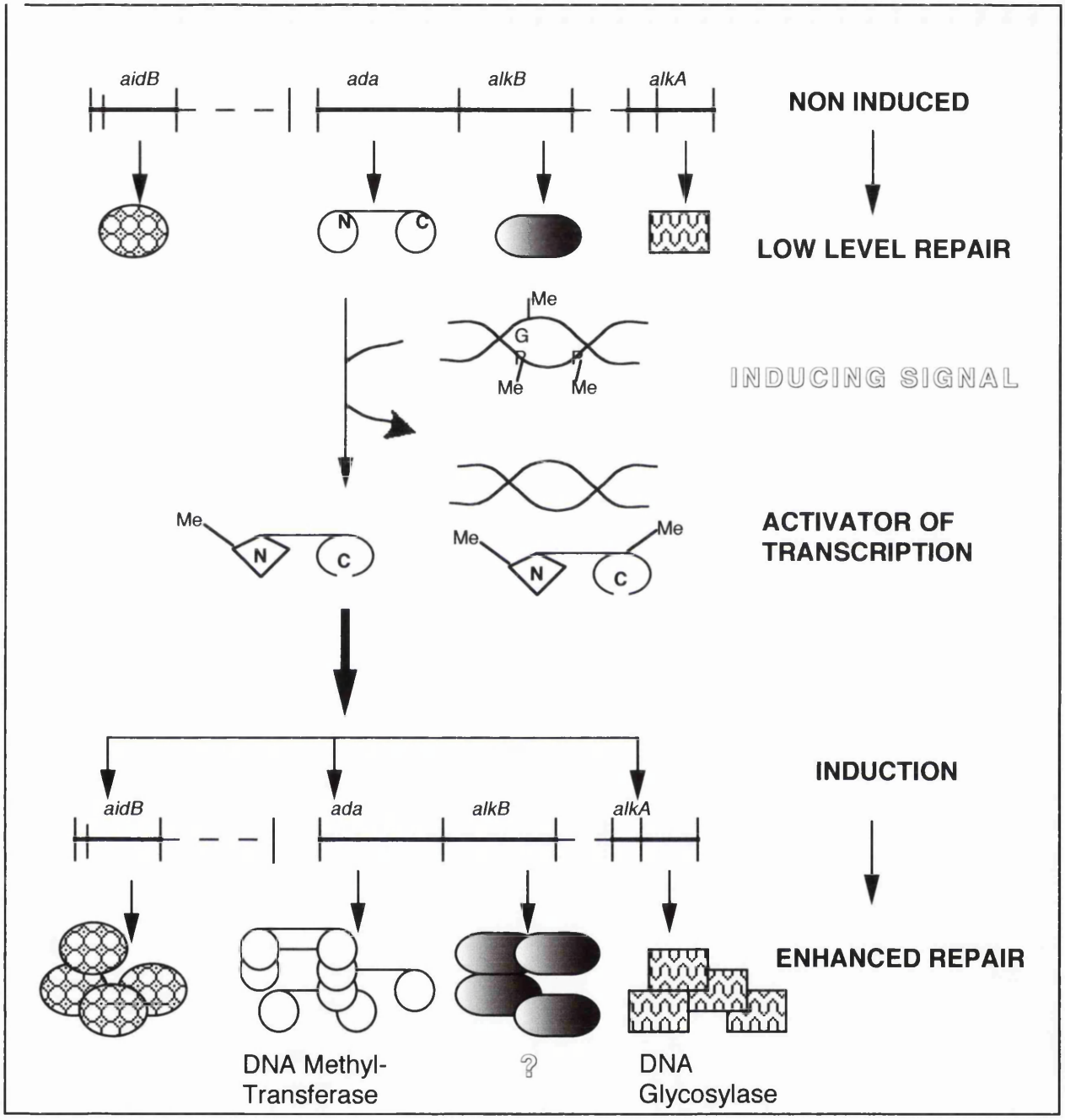


FIGURE 2: REGULATION OF THE ADAPTIVE RESPONSE OF *E. COLI* TO ALKYLATION DAMAGE

Exposure of *E.coli* to methylating agents causes DNA alkylation at several sites. Transfer of methyl groups to the Ada protein occur from P(Me) to a N-terminal cysteine (Cys-69), and O^6MeG to a C-terminal cysteine (Cys-321). Transferral to Cys-69 transforms Ada into a transcriptional activator of the response, causing increased expression of all four genes. [Adapted from: Lindahl *et al.*, 1988].

diastereoisomer of P(Me)s are repaired (McCarthy and Lindahl, 1985). Proteolytic cleavage of Ada into fragments identified sizes of the active domains. The 39kDa protein can be cleaved *in vitro* into a 19kDa product that contains the C- terminal cysteine residue, and thus the active site for O^6MeG and O^4MeT repair, and a 15kDa N- terminal product to contain the active Cys-69 for phosphotriester repair (Teo, 1987). On self methylation the Ada protein is able to display conformational changes, which could determine its active regulatory state (Bhattacharyya *et al.*, 1998; Pearl and Savva, 1995). Transfer of methyl groups from P(Me)s is the inducing signal for the adaptive response, and turns Ada into an activator of transcription causing increased expression of all four genes to deal with alkylation damage (Lindahl *et al.*, 1988; Teo *et al.*, 1986).

As part of the response AlkA is induced and encodes 3-methyladenine- DNA glycosylase activity. AlkA (DNA glycosylase II) is a 31kDa protein that accounts for 5 – 10% of total 3MeA- DNA glycosylase activity in unadapted i.e. non- induced cells, but upon induction of the adaptive response its activity increases approximately 20 fold. AlkA has broad range substrate specificity able to repair toxic lesions that block DNA replication, namely 3MeA, 3MeG, O^2MeC, O^2MeT. It also repairs the innocuous lesion 7MeG slowly. Action of AlkA is not product inhibited, and is important for repair of 3MeA, and some of the quantitatively minor yet biologically important methylated lesions (Evensen and Seeberg, 1982; Karran *et al.*, 1982).

The function of the 60kDa AidB protein is still unknown. Expression of *aidB* is *ada* gene regulated, but also shows an *ada*- independent induction mechanism in anaerobic or slightly acidic pH conditions. Methylated Ada (^{me}Ada) is able to bind the AidB promoter region and stimulate its transcription, although ten-fold higher concentrations are needed in comparison to induction of transcription of Ada by ^{me}Ada (Landini and Volkert, 1995). AidB

overexpression reduces the mutagenic affects of MNNG, and also in acidic conditions the cells become more resistant to the compound. AidB may therefore be involved in a detoxification pathway to reduce the affects of MNNG, but its exact function remains unknown (Landini *et al.*, 1994). AidB shows homology to several mammalian acyl coenzyme A dehydrogenases which are involved in energy production during fermentation and anaerobic metabolism. Thus, there may be a link here to its requirement in anaerobic growth and the resulting acidic conditions. The *rpoS* gene encodes a σ factor (σ^s) required for expression of a variety of genes expressed in stationary phase cultures. Although inactivation of the *rpoS* gene reduces *aidB* gene expression, *aidB* responds to the aeration state of a culture rather than stationary phase growth (Volkert *et al.*, 1994).

A further gene product induced as part of the adaptive response is *alkB*. AlkB is a 24kDa protein, but its function is yet to be determined. As this is the aim of the thesis work, the *alkB* gene product is described in detail in the next section (Section 1.4).

All the gene products described above are induced during the adaptive response of *E. coli* to alkylation damage. Another DNA methyltransferase, Ogt, and a DNA glycosylase called Tag (3-methyladenine- DNA glycosylase I) are constitutively expressed in cells. Ogt (19kDa) repairs similar lesions to the C-terminal domain of Ada. It can repair lesions O^6-ethylG, O^4MeT more rapidly than Ada, and O^6MeG at the same rate. It also deals with longer chain alkylation products that are known to be inefficient in inducing the adaptive response. Ogt does not repair P(Me)s and so this lesion remains the essential signal for induction of the adaptive response. The presence of Ogt enables repair of low levels of mutagenic and toxic lesions in the absence or before triggering the adaptive response (Margison *et al.*, 1990). Tag (21kDa) identified in 1978 is a DNA glycosylase that specifically repairs 3MeA. It exhibits a low

K_m for activity and is product inhibited unlike AlkA, suggesting its specific action towards the cytotoxic lesion 3MeA (Riazuddin and Lindahl, 1978).

Conservation of induction of the adaptive response is varied through evolution, but enzymes with similar functions as those induced in *E. coli* have been conserved. Bacteria other than *E. coli* that display the adaptive response include *Bacillus subtilis*, *Micrococcus luteus*, and *Bacillus thuringiensis*. The response has not been detected in microorganisms such as *Haemophilus influenza*, *Salmonella typhimurium* (weak response) or yeast *Saccharomyces cerevisiae* (Sedgwick and Vaughan, 1991; Vaughan *et al.*, 1991). Neither has the method of induction of the response been conserved through evolution to the mammalian system.

Bacillus subtilis cells exhibit an adaptive response. The cells possess Ada and AlkA homologues with equivalent functions but with a different genetic organisation. In *E. coli*, *ada* generates one gene product and forms an operon with the *alkB* gene. *B. subtilis* has two separate genes *adaA* and *adaB* that overlap by eleven base pairs and form a small operon. AdaA is 27kDa and is the methyl-phosphotriester- DNA methyltransferase, while the 22kDa AdaB encodes the O^6-methylguanine- DNA methyltransferase activity. Sequence similarity exists with the *E. coli ada* gene product indicating the importance of functional conservation. In the presence of alkylation damage the *B. subtilis adaA* gene product is sufficient for transcriptional activation of the response (Morohoshi *et al.*, 1990). An inducible AlkA encoding 3MeA- DNA glycosylase activity has also been found in this bacterium, but no AlkB or AidB proteins. Also constitutive Tag and Ogt like activity has been reported. The *alkA* gene was found to be upstream of the *ada* operon, and in the opposite orientation for transcription initiation. Its action is regulated by AdaA, but its activity is not primarily responsible for removal of all lethal lesions induced by alkylating agents such as MMS (Morohoshi *et al.*, 1993).

In *Salmonella typhimurium* Ada protein has been detected, but only a weakly induced adaptive response is associated. Repair of P(Me)s by the *Salmonella typhimurium* Ada protein was just as efficient as by the *E. coli* protein. Hence the lack of induction of the adaptive response in this species was attributed to Ada being a poor transcriptional activator (Vaughan and Sedgwick, 1991).

Lack of an adaptive response is apparent in many organisms. Evolution has dictated that yeast shows no conservation of the method of induction as in *E. coli* cells. *Saccharomyces cerevisiae* possess both O^6-methylguanine- DNA methyltransferase (*MGT1*) and 3-methyladenine- DNA glycosylase (*MAG*) activities. MGT1 levels are not increased in response to alkylation damage. In contrast induction of MAG occurs in *S. cerevisiae* in the presence of alkylation damage, UV and 4-nitroquinoline-1-oxide (4NQO). However, the method of induction is not the same as in *E. coli.* i.e. it is not regulated by MGT1, but it does require *de novo* protein synthesis (Chen and Samson, 1991; Xiao *et al.*, 1991).

In the mammalian system the adaptive response is not conserved, but enzymes of analogous function do exist to deal with alkylation damage. Mammalian O^6-alkylguanine- DNA transferase is approximately 22kDa, removes O^6MeG lesions and larger adducts that can form on the O^6- position of guanine. Efficiency in removing O^4MeT lesions is not as good as for the *E. coli ada* gene product (Pegg, 1990). The importance of the DNA methyltransferase is highlighted by a mouse knockout of the gene product *Mgmt* (methylguanine-DNA methyltransferase). These mice are viable but are cancer prone on exposure to alkylating agents (Kawate *et al.*, 1998). Cells devoid of DNA methyltransferase activity are known as Mer^- or Mex^- cells (Demple, 1990). Investigations using Mex^- Chinese hamster ovary cells (CHO) cell lines showed that inability to repair O^6-alkylguanine lesions triggered S- phase cell cycle arrest and apoptosis (Meikrantz *et al.*, 1998). An interesting link exists to

mismatch repair (MMR) in humans, when the normally non- lethal yet mutagenic lesion O^6MeG becomes a lethal lesion. During DNA replication unrepaired O^6MeG can be copied although incorrectly, due to its miscoding properties. MMR is then activated due to the presence of the mispaired base on the newly synthesised DNA strand. MMR excises the mismatched base, but the lesion O^6MeG still remains on the old DNA strand. Repeated futile cycles of repair result in lethality due to O^6MeG. Thus, in order to tolerate O^6MeG in DNA the cells would have to loose the mismatch corrective pathway (Karran and Hampson, 1996).

The 3-methyladenine- DNA glycosylase activity is conserved through evolution from *E. coli* (AlkA/ Tag), yeast (MAG in *Saccharomyces cerevisiae*), mouse (Aag), plants (aMAG in *Arabidopsis thaliana*) to humans (AAG). Human 3-methyladenine- DNA glycosylase (AAG) can remove lesions such as 3MeA, 3MeG, 7MeG, hypoxanthine, and ethenoadenine products from DNA. Lack of this enzyme in mouse knockouts is associated with a viable phenotype and normal development (Wyatt *et al.*, 1999), though lack of ability to repair the lesion 3MeA in cells exposed to alkylating agents can induce S- phase cell cycle arrest, sister chromatid exchange, chromosome gaps and breaks, and apoptosis (Engelward *et al.*, 1998).

Above are described the activities associated with the repair of alkylation damage, which prevents increased mutation frequencies and cell death by promutagenic or lethal lesions.

1.4 HISTORY OF ALKB

The *E. coli* genetic locus '*alk*' was first described in 1978 by Yamamoto *et al.* Replica plating, host cell reactivation experiments and enzyme activity assays were used to isolate and characterise the *E. coli* mutants sensitive to alkylating agents, but not to UV or gamma rays (Yamamoto *et al.*, 1978; Yamamoto and

Sekiguchi, 1979). Genetic mapping experiments showed that different chromosomal locations existed for the *alk* mutations, and in 1983 Kataoka *et al* identified the *alkB* genetic locus (*alkB22*) at 47 min on the *E. coli K-12* genetic map linked to the nalidixic acid resistance (*nalA/ gyrA*) marker. The *E. coli* strains carrying the *alkB* mutations were less capable of reactivating MMS treated lambda bacteriophage, showing an approximately two-fold decrease on comparison to the parent strain. This indicated that the mutant *alkB* was probably defective in repair of alkylated DNA. The identifying feature of the *alkB* mutant cells was their greater sensitivity to MMS than MNNG (Kataoka *et al.*, 1983). Molecular cloning in 1985 suggested that the *alkB* gene coded for a polypeptide of approximate molecular weight 27,000 Daltons, and that it was next to the *ada* gene on the *E. coli* chromosome. Thus a deletion of the *alkB* gene with a small portion of the *ada* gene abolished ability of the cells to induce the adaptive response (Kataoka and Sekiguchi, 1985). These analyses were followed up a year later with overexpression and purification of the AlkB protein. Amino acid composition with amino-terminal sequence analyses via automated Edman degradation, showed it to be comprised of 216 amino acids encoding a protein of molecular weight 23,900 Daltons (Kondo *et al.*, 1986). This procedure was the first to show AlkB's exact composition, and its predominant hydrophobicity. The nucleotide sequence of the *alkB* gene was also determined. Sequencing of the *alkB* gene product confirmed that it was next to the *ada* gene, and that the last nucleotide of the *ada* termination codon (TA<u>A</u>) overlapped with the first nucleotide of the *alkB* initiation codon (<u>A</u>TG). Gene expression studies using the intact *ada* gene preceding *alkB*, the *ada* gene minus its promoter region, or the *ada* promoter with a defective *ada* protein coding region, revealed that the *ada* and *alkB* genes constituted an operon. The expression of the *alkB* gene was cotranscribed with the *ada* gene, and was under control of the *ada* gene promoter itself (Kondo *et al.*, 1986).

Possible interactive functions of AlkB with other known gene products have

been investigated. The *alkA* and *alkB* gene products were reported to function independently, and so to act in different DNA repair pathways (Volkert and Hajec, 1991). *E.coli* AlkB protein was expressed in human cell lines, and was able to confer an alkylation resistant phenotype. The *alkB* gene was placed under control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. This promoter was used as it could promote transcription in *E.coli* and mammalian cells. The plasmid was transfected into two independent human HeLa cell lines. When AlkB protein was present, the cell lines showed resistance to alkylating agents, specifically to the S_N2 agents MMS and DMS. No resistance was seen on treatment with the S_N1 alkylating agents MNNG or MNU, or with a more complex alkylating agent BCNU. The degree of alkylation of the HeLa cells genome by DMS was found to be equal ± AlkB expression, producing the same number of total alkyl groups upon damaging DNA. Thus, AlkB did not degrade MMS. MMS also induces sister chromatid exchanges (SCE) within cells, and AlkB did not prevent SCE's from occurring. Overall, the experiments suggested a functional independence for AlkB action. It was concluded that its action was probably to directly remove damage from the DNA, or to process it in a manner which is not cytotoxic to the cell (Chen *et al.*, 1994).

After initial recognition of a gene in a bacterial system a question often asked is of its conservation through evolution, and homologues based on sequence homology have been reported for AlkB. Genes which can complement the AlkB phenotype, but which have no sequence homology have also been reported. In 1995 three such genes from yeast *Saccharomyces cerevisiae* were identified called *YFW1*, *YFW12* and *YFW16*. The complementing activity was specific to *alkB* mutants, as the yeast genes were not able to rescue *alkA tag*, *recA*, or *uvrB* mutant cells from MMS damage. YFW1 and YFW12 were highly serine and threonine rich, resembling membrane glycoproteins. The *YFW16* gene product was allelic with the *STE11* gene, a protein kinase. Hence the three

genes were able to rescue *alkB* mutants cells from alkylation damage, though not as efficiently as the *E. coli* AlkB protein itself (Wei *et al.*, 1995).

A year later a human homologue of AlkB was found in a database of expressed sequence tags (ESTs), and was the first true homologue to be described based on sequence homology (Wei *et al.*, 1996). ESTs are segments of sequence from cDNA clones that correspond to mRNA sequences (Adams *et al.*, 1991). An EST from a human synovial sarcoma cDNA library showed homology to the C-terminal half of *E. coli* AlkB, and was termed hABH. It showed 23% identity, and 52% similarity to the *E. coli* AlkB protein, and a small region existed showing a higher degree of conservation with 34% identity and 59% similarity. The homologue *hABH* mapped to chromosome 14 (14q24), and was found to be expressed in most human tissues, indicating a role as a house keeping gene. The expression of hABH protein in *E. coli alkB* mutant cells partially rescued the cells from death when exposed MMS. The rescue may have been only partial because of the difference of hABH to *E. coli* AlkB of an additional eight amino acids at the N- terminus. HABH is a 34kDa protein compared to the 24kDa AlkB in *E. coli*. Five insertions, forming two clusters within the hABH protein could be a result of evolutionary changes reflecting the complexity of higher eukaryotic systems. Also, *hABH* did not form an operon with its *ada* homologue MGMT which was located on a different chromosome. Thus there was no co- regulation of these genes as in *E. coli* (Wei *et al.*, 1996).

A bacterial homologue in *Caulobacter crescentus* shows 42% identity and 78% similarity to *E. coli* AlkB. This homologue was found in 1997 whilst cloning the heat shock genes of *C. crescentus*. The *C. crescentus* AlkB protein is 21 kDa, and a mutant of the gene shows MMS sensitivity. As in humans, there was no induction of an adaptive response, and *C. crescentus alkB* did not lie downstream from an *ada* gene homologue (Colombi and Gomes, 1997). During cell replication *C. crescentus* divides asymmetrically, first into a sessile stalked

cell and then to a motile swarmer cell. Only the stalk cell is able to carry out DNA replication (Marczynski *et al.*, 1990; Zweiger and Shapiro, 1994). An interesting property of the *C. crescentus* AlkB homologue was its temporal regulation. The AlkB levels increased during DNA replication i.e. at the stalked cell stages of *C. crescentus.* As damage by alkylating agents can occur at any time, it was hypothesised that increased levels of AlkB during replication could confer protection from the increased susceptibility to DNA damage at this time (Colombi and Gomes, 1997).

Shown in Figure 3 are homologues of *E. coli* AlkB. They include bacteria *Caulobacter crescentus* (78% similarity; 42% identity), yeast *Schizosaccharomyces pombe* (45% similarity; 30% identity), plant *Arabidopsis thaliana* (47% similarity; 39% identity), *Drosophila melanogaster* (35% similarity; 30% identity) [Personal communication – Dr. John Sgouros], and *Homo sapiens* (52% similarity; 23% identity). These percent similarities and identity were calculated using Bestfit from the Genetics Computer Group software suite 9.1 programme. Finally just reported is a novel hydrolase domain in AlkB which could offer insight into its function (Aravind *et al.*, 1999).

In summary described here are just under two decades of work aiming to understand the fundamental role of the *alkB* gene product. Still it remains a mystery, and in view of all the information gained over the years, the aim of this Ph.D. project is to further clarify the function of AlkB.

Figure 3: Alignment Of AlkB Homologues

Prokaryotic:
Bacteria: *Escherichia coli; Caulobacter crescentus*

Eukaryotic
Yeast: *Schizosaccharomyces pombe*
Plant: *Arabidopsis thaliana*
Fly: *Drosophila melanogaster*
Human: *Homo sapiens*

The sequences were aligned using clustalx and identical and similar sequences indicated using SeqVu 1.1


E. coli 1 0
C. crescentus 1 0
A. thaliana 1 65
S. pombe 1 49
H. sapiens 1 3
D. melanogaster 1 0
E. coli 1 40
C. crescentus 1 20
A. thaliana 66 131
S. pombe 50 115
H. sapiens 4 69
D. melanogaster 1 27
E. coli 41 88
C. crescentus 21 68
A. thaliana 132 197
S. pombe 116 161
H. sapiens 70 114
D. melanogaster 28 74
E. coli 89 152
C. crescentus 69 128
A. thaliana 198 263
S. pombe 162 226
H. sapiens 115 177
D. melanogaster 75 139
E. coli 153 195
C. crescentus 129 171
A. thaliana 264 324
S. pombe 227 266
H. sapiens 178 243
D. melanogaster 140 187
E. coli 196 216
C. crescentus 172 192
A. thaliana 325 354
S. pombe 267 278
H. sapiens 244 299
D. melanogaster 188 206

1.5 OVERVIEW OF OTHER DNA DAMAGE AND REPAIR

DNA damage, a major challenge and threat to stability of the genome, has to be repaired in an efficient and precise manner. Each human cell may undergo approximately 20,000 modifications in one day (Lindahl, 1993). This large number is indicative of the importance to maintain genomic stability by repairing the DNA modifications. Both exogenous and endogenous damage to DNA can occur. Environmental stresses include exposure of DNA to alkylating agents, ionising radiation, and UV light. Intracellular DNA damage includes DNA methylation, crosslinks, oxidation and strand breaks. Evolution has ensured conservation from bacteria to man of an array of DNA repair mechanisms to accurately deal with DNA damage to ensure survival of species. Using the *E. coli* bacterial system as a model, an overview of the different repair processes to deal with various types of DNA damage are described, mentioning their conservation in the eukaryotic mammalian system.

The base excision repair (BER) pathway removes non- bulky DNA lesions. These include deaminated bases such as deamination of cytosine to uracil, 5-methylcytosine to thymine, guanine to xanthine, and adenine to hypoxanthine; miscoding lesions able to produce base transition mutations. Reactive oxygen species from normal oxygen metabolism react with DNA to produce lesions such as 8-hydroxyguanine a mispairing lesion, thymine glycol a cytotoxic lesion and imidazole ring- opened derivatives of purines e.g. 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy), structures which can distort the DNA helix. Also depurinated and alkylated (e.g. 3MeA) DNA are dealt with by BER (Friedberg *et al.*, 1995; Wilson III and Thompson, 1997). *E. coli* has evolved around seven different DNA glycosylases to initiate removal of damage in the multi- step BER pathway (Sakumi and Sekiguchi, 1990). Initially damage is recognised and excised by a specific DNA glycosylase enzyme according to the

type of damage present, with some enzymes possessing a broad range substrate specificity. A few examples of these enzymes include uracil- DNA glycosylase (*ung*) for removal of uracil, formamidopyrimidine- DNA glycosylase (*fpg/ mutM*) for removal of FaPy, 7,8-dihdro-8-oxo-guanine and 5-hydroxycytosine, and thymine glycol- DNA glycosylase for removal of thymine glycol. Removal of the base lesion results in an apurinic or apyrimidinic (AP) site, which is then cleaved and repaired. AP- endonucleases cleave the phosphodiester backbone 5' to the AP site. *E. coli* exonuclease III (*xth*) or endonuclease IV (*nfo*) which also possess 3'-phosphodiesterase and 3'-phosphatase activities perform this function. The AP site can also be cleaved on the 3' side by the β-elimination mechanism of an AP-lyase. A 5'-deoxyribophosphodiesterase (*recJ*) removes the remaining deoxyribose- phosphate at the 5' end of the strand break. A one nucleotide gap results which is filled by *E. coli* DNA polymerase I (Pol I), and the DNA strand sealed by *E. coli* DNA ligase (Seeberg *et al.*, 1995).

The eukaryotic mammalian BER system follows a similar multi- step procedure with analogous enzymes for repair. A fundamental difference is that in mammals the BER pathway forks into two, the short patch repair pathway which involves single nucleotide replacement as in the prokaryotic system, or the long patch repair pathway replacing greater than one but less than fourteen nucleotides at a time (Frosina *et al.*, 1996). Short patch BER requires recognition by a specific DNA damage glycosylase. Following this, incision by the AP endonuclease (HAP1) occurs, and then gap filling and ligation with DNA polymerase β and DNA ligase III/ XRCC1 (Lindahl *et al.*, 1997). The long patch pathway requires additional factors on comparison to short patch repair, and is dependent on the *FEN1* and PCNA gene products. The FEN1 enzyme encodes a 5'-flap endonuclease to excise the flap like structure produced by strand displacement (Klungland and Lindahl, 1997). The long patch pathway can be further subdivided into the proliferating cell nuclear antigen (PCNA) stimulated, Polβ directed, or the PCNA dependent Polδ/ε

directed branches (Wilson III and Thompson, 1997). PCNA is an ubiquitous protein that can encircle DNA, and can interact with many proteins that play an essential role in eukaryotic nucleic acid metabolism (Jonsson *et al.*, 1998; Kelman and Hurwitz, 1998). Overall it is seen that BER has been intricately engineered to cope with a host of damage and has remained conserved through evolution.

Nucleotide excision repair (NER) of *E. coli* can deal with most types of damage for example UV induced pyrimidine dimers, and is not limited to damage recognition by glycosylases. Damage in *E. coli* is recognised by the UvrA protein, an ATPase which dimerises to form $UvrA_2$. On dimerization of UvrA, a DNA helicase UvrB is able to bind the damaged DNA causing local unwinding and displacing of the UvrA. UvrB bound to DNA allows UvrC to join, activating a dual incision reaction. UvrC bound to UvrB induces a 3' DNA incision approximately 5 bases away from the lesion, and the UvrC makes a DNA incision approximately 8 bases 5' to the lesion. The UvrD helicase is recruited to release UvrC and the DNA lesion contained in a fragment 12 - 13 nucleotides in length. DNA Pol I fills the gap and displaces the UvrB, and DNA ligase ligates the strand (Grossman and Thiagalingam, 1993; Sancar, 1996).

Monomerization of thymine dimers also occurs in *E. coli* by an enzyme activated by light called 'DNA photolyase (photoreactivating enzyme)'. It is a product of the *phr* gene and specifically repairs cyclobutane dimers in UV and also binds cisplatin- damaged DNA. Conservation of this enzyme in higher eukaryotes is absent, but the NER process to excise damage has been conserved (Sancar, 1996). Ability to reconstitute the repair pathway *in vitro* helped identify the human proteins involved (Wood *et al.*, 1988). Analogous proteins exist to perform similar functions as in NER of *E. coli.* In yeast these consist of the Rad family of proteins, and in humans a group of seven XP proteins designated XPA- XPG.

XPA binds DNA damage via its zinc finger domain, and to single strand binding protein RPA to further enhance its affinity to the damaged DNA. XPA also binds transcription factor IIH (TFIIH). TFIIH contains many subunits that include the XPD and XPB proteins. XPD and XPB have DNA unwinding helicase activity and open the DNA structure around the damaged site. XPA then binds XPF/ERCC1 a 5' DNA nuclease, and XPG a 3' DNA nuclease. Damage is incised in a similar way to the UvrC incision of *E. coli*. XPC stimulated by XPE is required for this incision, protecting and stabilising the pre- incision complex. A fragment of DNA containing the damage is released (~29 nucleotides) and Polε, PCNA, replication factor C (RFC) and DNA ligase I recruited to fill and seal the gap (Lehmann, 1995). It is interesting to note the overlap within the eukaryotic system of the last stages of NER and the long patch repair pathway of BER.

Mismatch repair (MMR) exists primarily to repair mismatched bases that arise during DNA replication. In *E. coli* adenines in GATC sequences are methylated by the enzyme Dam methylase. Newly synthesised DNA strands are briefly unmethylated to allow recognition of mismatched bases by the MMR pathway. Repair is initiated by protein MutS that recognises a mismatched base. MutL is recruited, and its binding to MutS activates MutH, a DNA endonuclease that causes incision on the unmodified DNA strand at a hemimethylated d(GATC) site. In the presence of MutU, a DNA helicase II (UvrD of NER), and a single stranded DNA exonuclease the region of the unmodified DNA strand spanning the GATC site and mismatch is excised. If a 5' incision to the lesion occurs exonuclease VII or RecJ excises the damage, but if a 3' incision is involved exonuclease I is used. DNA Pol III, single stranded binding protein (SSB), and DNA ligase finally fill the gapped DNA (Modrich, 1994). Distinct short patch MMR in *E. coli* can be subdivided into a base- specific MMR that resembles BER, the very short patch repair pathway (VSP) requiring VSR, an endonuclease that specifically repairs G-T and G-U mispairs, and the Mut-Y

dependent repair pathway which repairs A-C and A-G mismatches. MutY can also reverse damage of oxidised guanine. If 8-oxo-dGTP forms, MutT protein normally degrades this. If it gets incorporated into DNA opposite cytosine then action of the Fpg (MutM)- DNA glycosylase of BER can remove it. However if the DNA with the 8-oxoG is replicated, and adenine misincorporated then MutY an A-G-mismatch-DNA glycosylase is required to remove the adenine from the 8-oxoG- adenine mismatch (Kolodner, 1995).

MMR is conserved in eukaryotes showing protein homology from yeast to man. In the mammalian system homologues to the *E. coli* genes include hMSH2, hMSH3, hMLH1, hPMS1 (Post- meiotic segregation) and hPMS2. Humans are capable of short patch MMR resulting in excision of one or a few nucleotides, and long patch MMR where a ~90- 170 nucleotide excision can occur and no sequence specificity is required. The MutS homologues are MSH2 and MSH6 which form a dimer MutSα that binds specific base/ base mismatches. MSH2 and MSH3 can also form a dimer called MutSβ which binds and removes extrahelical nucleotides. The MutL homologues are MLH1 and PMS2 which form a dimer MutLα. PMS1 can bind MLH1 as well. Mismatches are excised and polymerase δ/ε, PCNA and ligases used to fill the gap (Modrich, 1997; Yu *et al.*, 1999).

Recombination is important in repair of DNA breaks resulting from damage such as ionising radiation (IR), as well as for generating genetic diversity in cells by homologous recombination. Recombinational repair requires alignment of two parental DNA molecules, followed by DNA single strand invasion and formation of a four- strand branched structure called a Holliday junction. DNA exchange occurs, with final resolution of the recombinant DNA molecule. Homologous recombination requires RecA to mediate the process by annealing two homologous DNA molecules, of which one is single stranded or partially single stranded. Other proteins involved are the RecBCD enzyme, a DNA

helicase and nuclease which unwinds the DNA until a cleavage hot spot chi (*X*) is reached for strand exchange, RuvAB for binding Holliday junctions, RecG for promoting branch migration and RuvC for resolution of the intermediates. A whole host of other Rec proteins are needed which include RecE, RecF, RecJ, RecN, RecQ, RecR and RecT that have various DNA metabolic activities including DNA polymerase, topoisomerase, ligase and binding activities. In contrast recombination can also occur via an alternative route of single- strand annealing, and does not involve formation of an intermediate Holliday junction. The double strand break is processed by a helicase or nuclease. Regions of homologous SS DNA are exposed and then bound to a protein that is capable of renaturing the homologous DNA formed. This alternative process happens rarely but is more analogous to the repair of DNA breaks in eukaryotes (Eggleston and West, 1996; West, 1997).

Apart from the fundamental RecA protein, recombinational repair has not been conserved per se, although mechanisms to repair strand breaks do occur in the mammalian system. Two pathways exist in the mammalian system, and include homologous recombination (HR) as in *E. coli*, and non- homologous end joining (NHEJ). NHEJ forms the major pathway for repair in humans, and unlike HR does not require homologous sequences elsewhere in the genome (Takata *et al.*, 1998; Taylor and Lehmann, 1998).

The RecA homologue in mammals is hRAD51B, and HR occurs by a similar mechanism to that in *E. coli*. HRAD52 induces the process of hRAD51B strand directed exchange by promoting the annealing of complementary SS DNA, and by binding to the single strand binding protein RPA (Kanaar *et al.*, 1998; Yu *et al.*, 1999). In comparison NHEJ does not involve strand exchange. NHEJ depends on a DNA- dependent protein kinase (DNA-PK) that has a regulatory heterodimeric subunit 'Ku', and a kinase catalytic domain DNA-PK_{cs}. The requirement for these activities was recognised by studying ionising radiation

sensitive cell lines designated XRCC1- 10, (X-ray sensitive cross complementing) (Jeggo, 1997). In humans the proteins Ku86 (XRCC5) and Ku70 (XRCC6) form the subunits of Ku that recognise the double strand break in DNA, and on complexing with the DNA-PK_{cs} catalytic subunit (XRCC7) phosphorylation activity is activated. XRCC4 is a nuclear substrate for DNA-PK, and associated with ligase IV is recruited to ligate the DNA ends to repair the double- strand break (Yu *et al.*, 1999). This mechanism of DNA strand break repair is also required in the process of V(D)J recombination in eukaryotes, an example of site specific recombination in the development of B and T cells of the immune system (Jackson and Jeggo, 1995).

An enzyme not yet discussed is poly (ADP- ribose) polymerase (PARP). Although not linked directly to a DNA repair pathway, PARP acts as a sensor towards nicks in DNA especially those occurring in strand interruptions during DNA replication (Lindahl *et al.*, 1995).

The SOS response of *E. coli* is a mutagenic pathway induced when error free processes fail to repair DNA damage. It allows lesions to be bypassed by misincorporation of a nucleotide opposite the lesion, followed by continued DNA replication.

SOS is a regulon of over twenty unlinked genes. The main gene products required for the mutagenic response are *recA*, *lexA*, *umuD* and *umuC*. Presence of DNA damage causes RecA to be activated by binding to damaged SS DNA regions. Activated RecA (RecA*) functions as a coprotease, and results in stimulation of autodigestion activity of LexA, normally the repressor of the SOS response. LexA autodigestion results in induction of the UmuDC operon and all the other LexA regulated genes. LexA binds the area of RecA which is normally the binding site for double stranded DNA during recombinational repair. Hence this repair process is switched off. UmuD shares homology with

the carboxyl- terminus of LexA which contains the autodigestive activity. Thus, UmuD is stimulated by RecA* to undergo cleavage as well to UmuD'. UmuD' dimerises to form UmuD'$_2$, and interacts with UmuC to form UmuD'$_2$C, which is the active complex for translesional synthesis to occur. GroEL and GroES are involved as chaperons of this process by maintaining the stability of UmuC until it complexes with UmuD'$_2$ (Smith and Walker, 1998). It has recently been reported that uncleaved UmuDC plays a part in cell cycle regulation by delaying DNA replication to allow time for accurate DNA repair to initially occur (Opperman *et al.*, 1999). Also the UmuC is now recognised as the polymerase (Pol V) involved during bypass (Tang *et al.*, 1999). Other polymerases for translesional DNA synthesis occur in *E. coli* such as DinB (Pol IV) for replication past misaligned DNA templates. Conservation of SOS to the mammalian system is still poorly understood, although homologues to the *E. coli* DNA polymerases exist to replicate past damaged DNA (reviewed by, Woodgate, 1999).

Described above are the *E. coli* DNA repair pathways, and their conservation through evolution to mammalian systems. It is interesting to note their overlapping functions, and so DNA repair events are not mutually exclusive, indicating the significance of repair to maintain genomic stability and survival of species (Cox, 1995). Either direct conservation of proteins through evolution has occurred, or species have evolved analogous proteins with similar functions. As the mammalian system is more complex, generally there exists more than one protein to perform a similar function on comparison to the simpler bacterial system.

The relevance of the repair pathways are verified by constructing knockout mice of specific genes of DNA repair. Various affects have been seen. For example mutating Polβ or ligase I of BER resulted in embryonic lethal phenotypes, whereas knocking out genes of NER produced viable mice but

with increased UV sensitivity and a high predisposition to skin cancer. Knockout mice defective in MMR and double strand break repair as well as others have been investigated (reviewed by Friedberg and Meira, 1999).

Clinical symptoms in higher organisms resulting from lack of DNA repair activity range from severe symptoms to a higher predisposition to cancer to, even death (Yu *et al.*, 1999). This can be highlighted by some well-documented clinical symptoms due to lack of DNA repair. Indeed it is from these symptoms that some repair pathways came to light. Noted examples include a deficiency of genes of NER that can lead to xeroderma pigmentosum (XP), Cockayne syndrome (CS) or Trichothiodystrophy (TTD). Xeroderma pigmentosum patients show clinical skin disorders, neurological abnormalities and are prone to skin cancer. Related deficiencies lead to CS and TTD but with different clinical features. People with CS show loss of adipose tissue, mental retardation, dwarfism with gait defects, cataracts, retinal atrophy and acute sun sensitivity. TTD patients show similar features to CS patients, but the defining feature is that they have sulphur deficient brittle hair. TTD represents a transcription syndrome, a result of mutations in the TFIIH subunit (Lehmann, 1998). MMR deficiencies give rise to hereditary nonpolyposis colon cancer (HNPCC) characterised by an early age onset of cancer (Kolodner, 1995). Syndromes resulting from a defect in double stand break repair include Nijmegan breakage syndrome (NBS) and Ataxia telangiectasia (AT). AT features are generally more striking compared to those of NBS, but both share features of radiosensitivity, increased predisposition to malignancy, immunodeficiency and chromosomal instability (Digweed *et al.*, 1999). Fanconi anaemia (FA) another clinical syndrome is under investigation at present. Cell lines from FA patients are hypersensitive to DNA cross- linking agents, but the exact link to DNA repair is still unclear (Barnes *et al.*, 1993; Buchwald and Moustacchi, 1998). These are a few of the known examples of clinical phenotypes that can arise from a deficiency of DNA repair mechanisms.

They highlight the need for DNA repair to prevent cell death and a high incidence of cancer.

1.6 AIM

To identify functional roles for the *alkB* gene product involved in the adaptive response of *E. coli* to alkylation damage.

Chapter 2

CHAPTER 2

Materials & Methods

All experiments have been carried out at least three times with reproducible results. The figures and tables show representative experiments.

2.1 MATERIALS

All chemicals and enzymes used were from laboratory stocks, or from Amersham Life Sciences, BDH laboratory supplies, Bio-Rad, Boehringer Mannheim, Dako, Fischer Scientific International, Fisons, Millipore, National Diagnostics, NEN Life Science Products, New England Biolabs, Packard, Pharmacia Biotech, Pierce, Sigma- Aldrich, Stratagene or USB, unless otherwise stated.

2.2 *E. COLI* STRAINS AND PLASMIDS

The *E. coli* K12 strains and plasmids used are presented in Table 2.

2.3 MEDIA FOR GROWING CELL CULTURES

Minimal media (supplemented with casamino acids unless otherwise stated): Minimal media was made by mixing 1 mM $MgSO_4$, 1x M9 salts, 0.2% glucose, 0.2% casein acid hydrolysate (Hy- case- amino, Sigma- Aldrich) and 2 μg/ ml thiamine hydrochloride (vitamin B1) (Miller, 1992).

The 1x M9 salts (pH7), a laboratory stock contained anhydrous di-sodium hydrogen orthophosphate, (Na_2HPO_4, 0.04M), potassium dihydrogen orthophosphate (KH_2PO_4, 0.02M), NaCl (0.009M), and ammonium chloride (NH_4Cl, 0.02M).

Luria – Bertani (LB) media: LB, a laboratory stock contained 10g bacto-tryptone, 5g bacto yeast extract, 10g NaCl per litre adjusted to pH7 using sodium hydroxide (NaOH) (Maniatis *et al.*, 1982).

STRAIN	GENOTYPE	SOURCE
AB1157	*argE3 hisG4 leuB6 Δ(gpt-proA)62 thr-1 ara-14 galK2 lacY1 mtl-1 xylA5 thi-1 rpsL31 glnV44 tsx-33 rfbD1 mgl-51 kdgK51*	Laboratory Stock
AB1886	as AB1157 but *uvrA6*	P. Howard-Flanders
BS87	as AB1157 but *alkB117*::Tn3	(Sedgwick, 1992)
BS141	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 /AB1157	XL1-Blue x AB1157
BS143	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 /BS87	XL1-Blue x BS87
BS145	as AB1886 but *alkB117::Tn3*	P1(BS87) x AB1886
BS146	as AB1157 but *Δ(umuDC)595::cat*	P1(RW120) x AB1157
BS147	as BS87 but *Δ(umuDC)595::cat*	P1(RW120) x BS87
BS152	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 /AB1886	XL1-Blue x AB1886
BS153	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 /BS145	XL1-Blue x BS145
BS154	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 /BS146	XL1-Blue x BS146
BS155	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 /BS147	XL1-Blue x BS147
BS163	as AB1157 but *mutS215::Tn10*	Laboratory Stock
BS164	as BS87 but *mutS15::Tn10*	Laboratory Stock
CC101 - CC106	*ara* $\Delta(lac\ proB)_{XIII}$ / F'*lacI*^{q}*Z proB*$^+$	(Cupples and Miller, 1989)
GC4803	as AB1157 but X::Tn5 *tagA1 alkA1*	(Boiteux *et al.*, 1984)
HK80	as AB115 7 but *nalA*	(Kataoka *et al.*, 1983)
HK82	as AB115 7 but *nalA alkB22*	(Kataoka *et al.*, 1983)
MS23	as AB1157 but *alkA1 his*$^+$	(Yamamoto and Sekiguchi, 1979)
PT11	CC102 but Δ(*ada-alkB25*)::Cam^r *ogt-1*::Kan^r	(Taverna and Sedgwick, 1996)
RW120	as AB1157 but *sulA211Δ(umuDC)595::cat*	R.Woodgate

STRAIN	GENOTYPE	SOURCE
RW202	*trpE65 lon-11 sulA1 Δ(srlR-recA)306*::Tn10	R.Woodgate
SD1	as GC4803 but *alkB117*::Tn3	P1(BS87) x GC4803
SD4	AB1157 but *Δ(srlR-recA)306*::Tn10	P1(RW202) x AB1157
SD5	BS87 but *Δ(srlR-recA)306*::Tn10	P1(RW202) x BS87
SD6	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn5/BS163	XL1-BlueMRF'kan x BS163
SD7	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn5/BS164	XL1-BlueMRF'kan x BS164
SD8	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn5/SD4	XL1-BlueMRF'kan x SD4
SD9	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn5/SD5	XL1-BlueMRF'kan x SD5
SD11-16	CC101-CC106 but *alkB117*::Tn3	P1(BS87) x CC102-CC106
XL1-Blue (Tet)	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn10 */supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*	Stratagene
XL1-Blue MRF' (Kan)	F' *proAB*$^+$ *lacI*^{Q} *lacZΔM15* Tn5 */supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*	Stratagene
VECTOR		
PET15b	Ampicillin resistant vector, 5.71Kb	Novagen

TABLE 2: *E. COLI K12* STRAINS AND PLASMIDS

2.4 MMS CONCENTRATION GRADIENT IN A L-AGAR PLATE

MMS gradient plates were prepared by mixing 25 µl of 100% MMS stock solution, (Sigma- Aldrich) with 50 ml molten agar in a 10 cm square plate. The plate was cooled at an angle to create a wedge of solid agar. Once set, another 50 ml of L- agar was poured on top to form an even surface and an increasing concentration of MMS from 0 - 5.9 mM (0 - 0.05%). The plate was dried inverted for 1.5 hours at room temperature. Various *E. coli* strains (10 µl) grown in minimal medium to A_{450} 0.4 were streaked across the L- agar plate, and incubated overnight at 37°C. Survival of the various strains was noted by the degree of growth across the MMS gradient.

2.5 MONITORING REPAIR OF DNA STRAND BREAKS *IN VIVO*

E. coli strains HK80 and HK82 carrying plasmid pET15b (5.71 Kb) were grown at 37°C in minimal medium containing carbenicillin (50 µg/ ml, Sigma-Aldrich) to A_{450} 0.5. The carbenicillin was present to maintain the pET15b plasmid. Aliquots (20 ml) were exposed to increasing concentrations of MMS (0 - 59 mM (0 - 0.5%)) for 20 min at 37°C, and the cells then pelleted. Plasmid DNA was extracted using the QIAamp Tissue Kit (Qiagen) (Yakes *et al.*, 1996). The plasmid DNA was analysed for its open circular (OC) and closed circular (CC) forms by agarose gel electrophoresis (0.7% gel). The bands on the gel were quantitated using a Molecular Dynamics Computing Densitometer.

Survival of HK80/ pET15b, and HK82/ pET15b was monitored at the same time. Prior to pelleting the cells, 100 µl of each strain was taken, and various dilutions plated on L- agar plates. Survival of each strain was monitored by colony counting after overnight incubation at 37°C.

2.6 STRAIN CONSTRUCTION BY P1 MEDIATED TRANSDUCTION

Transduction, the transfer of genetic material by viral infection was carried out to create strains SD1, 4, 5 and 11 – 16. A temperature sensitive variant of the bacteriophage P1 was used called P1*clr100* (mutation of the P1 repressor), encoding a chloramphenicol (CAP) resistance gene (Miller, 1992; Silhavy *et al.*, 1984).

2.6.1 P1*clr100* LYSOGENS OF DONORS

P1*clr100* lysogens of *E. coli* strains BS87 (*alkB117::Tn3*) and RW202 (*Δ(srlR-recA)360::Tn10*) were made. The strains were grown in LB media to late log phase A_{600} ~1.3. $CaCl_2$ required for P1*clr100* absorption was added to a final concentration (FC) of 0.01M. Fifty µl of varying dilutions (10^{-3} – 10^{-4}) of P1*clr100* lysate were added to 0.5 ml of cells, and allowed to stand for 30 min at 30°C. 10 µl and 100 µl volumes were plated onto L- agar plates containing thymine (20 µg/ ml) and CAP (20 µg/ ml), and incubated overnight at 30°C. Colonies were picked and restreaked onto L- agar plates (CAP/ thymine). Tetracycline (15 µg/ ml, Sigma - Aldrich) was also added to the plates in the case of RW202/ P1*clr100* colonies.

The plates were left overnight at 30°C. High inoculum cultures were grown the next day in LB medium containing CAP (20 µg/ ml) at 30°C. The cultures were grown to A_{600} ~1.1, and then frozen after addition of 20% glycerol for storage at -80°C.

2.6.2 P1*clr100* LYSATES

Cultures of BS87/ P1*clr100* and RW202/ P1*clr100* lysogens were grown in LB medium containing CAP (20 µg/ ml) and 10 mM $MgSO_4$ to help P1*clr100*

growth, to mid log phase A_{600} 0.3, as cultures too dense do not allow cell lysis. Two ml of cells were then transferred to 42°C and aerated for 35 min to induce P1*clr100* replication. Growth of the cultures was continued at 37°C for 60 min to allow cell lysis. The cultures started to clear over time. 0.01 x volume of chloroform ($CHCl_3$) was added to each cell culture and agitated for 5 min, to allow all cells to lyse to completion. The lysed cultures were centrifuged at 10,000g for 10 min to pellet the cell debris. The supernatant i.e. P1 lysates of BS87 and RW202, were stored with 0.01 x volume $CHCl_3$ at 4°C.

2.6.3 P1*clr100* TRANSDUCTIONS

Transductions were carried out to transfer the donor bacterial DNA packaged in the P1 phage to the recipients. DNA from BS87 was transferred to recipient GC4803 (*tagA1 alkA1*) to create a triple mutant SD1 (*tagA1 alkA1 alkB117::Tn3*) for use in experiments section 2.7. It was also used to create strains SD11 – 16 (CC101 *alkB117::Tn3* – CC106 *alkB117::Tn3*) for experiments in section 2.15. DNA from RW202 was transferred to recipients AB1157 (wild type) and BS87 to create strains SD4 (as AB1157 but *Δ(srlR-recA)306::Tn10*), and SD5 (*alkB117::Tn3 Δ(srlR-recA)306::Tn10*) for use in experiments sections 2.8 and 2.9.

The recipient strains were grown in LB medium at 37°C to A_{600} 0.9. $CaCl_2$ was added to 0.01 M. Varying amounts (10 µl and 100 µl) of the P1*clr100* lysates were added to 1 ml of recipient cell aliquots, and incubated at 42°C for 30 min to prevent lysogen formation. Sodium citrate (0.05 M, pH 7) was then added to chelate the $CaCl_2$. The cells were spun, and washed in LB medium containing 0.05 M sodium citrate, and finally re-suspended in 0.1 ml LB/ 0.05 M sodium citrate. Cells were plated (10 µl and 90 µl) onto selective L- agar plates containing sodium citrate (0.05 M) and thymine (20 µg/ ml). For *Tn3* the selective medium contained carbenicillin at 50 µg/ ml, and for *Tn10* tetracycline at 15 µg/ ml. Plates were incubated at 42°C overnight. The next day

colonies were restreaked and incubated again at 42°C. Stocks were made by picking and growing colonies in LB medium containing the appropriate antibiotic, and frozen at -80°C in 20% glycerol. New strains were checked by plating on L- agar plates containing carbenicillin, tetracycline, or CAP, and incubating at 37°C or 42°C. No growth was expected on the CAP plates and no cell lysis should occur on the plates incubated at 42°C unless P1*clr100* remained.

2.7 SENSITIVITY OF *E. COLI* STRAINS TO $MeOSO_2(CH_2)_2$-LEX AND MMS

E. coli strains were grown in minimal medium to A_{450} 0.5. One ml of cells were treated with increasing doses of MMS (0 - 11.8 mM (0 - 0.1%)) for 20 min, or $MeOSO_2(CH_2)_2$-Lex (0 mM – 0.15 mM, from B. Gold, USA) for 2 hours, both at 37°C. The $MeOSO_2(CH_2)_2$-Lex was dissolved in 95% ethanol to a final concentration of 2 mM by shaking at room temperature for 40 min, and then used immediately. After treatment the cells were serially diluted in 1x M9 salts containing 1 mM $MgSO_4$ and plated onto L- agar plates. Survival of each strain was monitored by colony counting after overnight incubation at 37°C.

2.8 F' TRANSFER TO CREATE STRAINS SD6 – 9

F' *proAB*$^+$ *lacI*^{Q} *lacZDΔM15 Tn5* factor was transferred from XL1- Blue MRF' Kan^r (Stratagene) into strains BS163 (as AB1157 but *ΔmutS 215::Tn10*), BS164 (*alkB117::Tn3 mutS 215::Tn10*), SD4 (as AB1157 but *Δ(srlR-recA)306::Tn10*), and SD5 (*alkB117::Tn3 Δ(srlR-recA)306::Tn10*) to create F' strains SD6, SD7, SD8 and SD9 respectively for use in experiments section 2.9.

E. coli strains BS163, BS164, SD4 and SD5 were grown in LB media and XL1-Blue MRF' in LB medium containing kanamycin (40 μg/ ml). At A_{600}, 0.5 ml of F' strain was mixed with 2 ml of the recipient strains, and left to stand at 37°C for 15 min. The cells were vortexed hard to break the mating strains apart, and

then serially diluted (10^{-2} – 10^{-4}) in 1x M9 salts containing 1 mM $MgSO_4$. 100 µl of each strain was plated onto L- agar plates containing kanamycin (40 µg/ ml) and tetracycline (15 µg/ ml), and left overnight at 37°C to select for strains carrying the F' factor. Colonies were picked and restreaked onto L- agar plates containing kanamycin and tetracycline, and left again at 37°C. The colonies were restreaked once more, and high inoculum cultures grown and stored in 20% glycerol, -80°C.

N.B. F' *proAB*$^+$ *lacI*^{Q} *lacZDΔM15 Tn10* (Tet^r) factor was transferred from XL1-Blue (Stratagene), when creating other *E. coli* F' strains that were not tetracycline resistant themselves.

2.9 BACTERIOPHAGE DNA HOST CELL REACTIVATION ANALYSES

2.9.1 SURVIVAL OF SS AND DS DNA BACTERIOPHAGE TREATED WITH MMS

Bacteriophage lysates M13mp18 (laboratory stock) and lambda (λ_{gv}) were laboratory stocks.

E. coli strains BS141 (AB1157/ F') and BS143 (*alkB117::Tn3*/ F') were grown in LB medium containing tetracycline (15 µg/ ml) to maintain for the F' factor. M13mp18 (8.4 x 10^{10} pfu/ ml) was diluted 100 fold in 1x M9 salts containing 1 mM $MgSO_4$. One hundred µl of M13mp18 was mixed with an equal volume of MMS (0 – 20 mM) dissolved in the same medium, incubated for 30 min at 30°C, and then serially diluted. The M13mp18 was titered for survival by infecting 100 µl of *E. coli* strains at A_{600} 0.6 with various phage dilutions. The cells and phage were mixed in 3 ml molten LB soft agar and plated onto L- agar plates. Plates were incubated overnight at 37°C, and monitored for survival by plaque formation. Similarly survival of MMS treated bacteriophage λ_{gv} (4 x10^{11} pfu/ ml, for 30 min, 37°C) was monitored in AB1157 and BS87 *E. coli* strains.

2.9.2 SURVIVAL OF SS DNA BACTERIOPHAGE TREATED WITH OTHER DNA DAMAGING AGENTS

Survival of M13mp18 after treatment with other DNA damaging agents was also monitored. DMS (0 – 1 mM, Sigma – Aldrich), MeI (0 – 225 mM, Sigma – Aldrich), and MNU (a gift from P. Swann, UK) were diluted in the same way as MMS, and used immediately to treat M13mp18 for 30 min at 30°C. For treatment with gamma irradiation, 200 µl M13mp18 (4 x 10^8 pfu/ ml) were exposed for increasing times to a CSl15 – 137 – Cs source irradiator emitting at 2.71 Gy/ min. The final doses were 0 – 500 Gy. After treatment with the different damaging agents the M13mp18 was diluted, and infected in *E. coli* strains BS141 and BS143. M13mp18 was titered for survival by plaque formation, by plating on L- agar plates and incubating overnight at 37°C.

2.9.3 SURVIVAL OF MMS TREATED SS DNA BACTERIOPHAGE IN VARIOUS *E. COLI* MUTANTS

M13mp18 was treated with increasing concentrations of MMS (0 – 20 mM) for 30 min at 30°C in the same way as in section 2.9.1, and infected into the *E. coli* F' strains. The *E. coli* F' strains (BS141, BS143, BS152 – 155, SD6 – 9) were grown in LB media containing 15 µg/ml tetracycline. The strains SD6 – 9 required kanamycin (40 µg/ ml) to select for the F' factor. The M13mp18 was titered for survival by monitoring plaque formation after overnight incubation at 37°C on L-agar plates.

2.10 SENSITIVITY OF *E. COLI* EXPONENTIAL AND STATIONARY PHASE CELLS TO MMS

Wild type (AB1157) and *alkB* (BS87) cells were grown in minimal media to exponential phase A_{450} 0.5 or for a further 16 h to stationary phase A_{450} 1.1. One ml of cells were treated with increasing doses of MMS (0 - 47.2 mM (0 - 0.4%)) for 20 min at 37°C. After treatment the cells were diluted in 1x M9 salts containing 1 mM $MgSO_4$, and plated onto L- agar plates. Survival of the two

strains was recorded by colony counting after overnight incubation at 37°C.

2.11 BINDING OF PURIFIED ALKB PROTEIN TO SS AND DS STRANDED DNA CELLULOSE

A slurry of native (DS) or denatured (SS) DNA cellulose (Pharmacia Biotech, binding capacity 10 mg protein/ ml DNA cellulose), was placed into an Eppendorf tube. The DNA cellulose was washed 3x with 100 µl load buffer (0.07 M Hepes – KOH pH 7.8, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 5% glycerol). After the final wash the DNA cellulose bed had a 30 µl volume. Three µg (125 pmol) purified AlkB protein, (laboratory stock) in 100 µl load buffer was added to the DNA cellulose matrix, and incubated at 0°C for 30 min. The DNA cellulose was spun and the supernatant collected. This was termed the prewash, and contained unbound AlkB protein. The DNA cellulose was then washed with 50 µl of load buffer and increasing NaCl concentrations (0–2.5 M).

Using a slot – blot micro sample filtration manifold from Schleicher and Schuell, varying volumes of each wash elutant were loaded onto the nitrocellulose filter membrane. 0.2 µl, 0.5 µl, 1 µl, 2 µl, and 3 µl volumes were loaded per NaCl wash in a total volume of 10 µl. This corresponded to amounts 0.01 µg (0.4 pmol) [{0.2/50 µl} x 3 µg AlkB], 0.03 µg (1.25 pmol), 0.06 µg (2.5 pmol), 0.12 µg (5 pmol) and 0.18 µg (7.5 pmol) respectively of the 3 µg AlkB loaded. Double volumes were loaded for the prewash fraction. An AlkB titration was also carried out to correspond to the µg amounts of protein loaded in each elutant wash, so that the intensity and hence recovery of each band could be compared directly.

Once the samples had been filtered through, the nitrocellulose filter membrane (Schleicher and Shuell nitrocellulose) was blocked in 5% Cadbury's Marvel

dissolved in PBSA (0.1 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, laboratory stock) for 1 hour at room temperature, and then rinsed 3x in PBSA. The membrane was exposed overnight at 4°C to primary polyclonal antibody that had been raised in rabbits against the whole of the AlkB protein, (1/ 200 dilution in PBSA containing 10% Fetal Calf Serum (FCS), 0.1 % Nonidet P40). The membrane was then rinsed in PBSA, and washed three times for 10 min in PBSA containing 1% Nonidet P40 (BDH). The nitrocellulose was exposed (3 h, room temperature) to a secondary antibody of a peroxidase–conjugated swine anti- rabbit immunoglobulin (1/ 500 dilution, Dako in PBSA containing 10% FCS). The membrane was washed again, and stained using 4-chloro-1-naphthol tablets (4CIN, Sigma- Aldrich). One tablet (30 mg) of 4CIN in 10 ml methanol was mixed with 15 µl hydrogen peroxide in 30 ml PBSA, and poured onto the membrane immediately. 4CIN is a horseradish peroxidase substrate that produces an insoluble blue end product that allowed detection of the antibody complex bound to AlkB protein.

2.12 OLIGONUCLEOTIDES USED FOR DNA GEL SHIFT AND FILTER BINDING ASSAYS

2.12.1 SYNTHESIS OF OLIGONUCLEOTIDES

Oligonucleotides (Table 3) were synthesised by a commercial DNA synthesiser using cyanoethyl chemistry on a 0.04 µM scale. Each oligonucleotide was dissolved in 0.3 M sodium acetate pH 5.2, 10 mM $MgCl_2$, and precipitated with three volumes 100% cold ethanol. After overnight incubation at –20°C, each oligonucleotide was washed twice in 80% cold ethanol, dried and then redissolved in 10mM Tris HCl, pH 8. The concentration of each oligonucleotide was estimated from its A_{260} reading using a spectrophotometer (Ultrospec II, LKB, Biochrom). An A_{260} of 1 corresponds to about 37 µg/ ml DNA.

2.12.2 5' END LABELLING OF OLIGONUCLEOTIDES

Oligonucleotides were 5' end labelled using T4 polynucleotide kinase (New England Biolabs). Oligonucleotides (50 ng/ µl) were incubated with T4 polynucleotide kinase buffer (New England Biolabs), T4 polynucleotide kinase (10 units), γ-^{32}P- ATP (20 µCi, NEN) for 30 min at 37°C. The labelling reaction was terminated by incubation at 65°C, 5 min. The reaction volume was made up to 100 µl with 10 mM Tris HCl, pH 8 and run through a Sephadex G-50 column to remove any unincorporated label.

2.12.3 ANNEALING OLIGONUCLEOTIDES TO GIVE DS DNA

After the labelling reaction but before the Sephadex G-50 step, a complementary oligonucleotide was added to the above reaction mixture in a ratio of 2:1 (complementary: labelled oligonucleotide). The mixture was heat treated at 95°C, 2 min, and then placed in a beaker containing boiling water and allowed to cool slowly to room temperature for the annealing process to occur. The reaction volume was made up to 100 µl and run through a Sephadex G-50 column. A summary of all oligonucleotides (SS and DS) to be used for sections 2.13 and 2.14 are presented in Table 4.

2.12.4 MMS TREATMENT OF OLIGONUCLEOTIDES

Oligonucleotides were treated with 300 mM MMS in a final volume of 50 µl, incubated at 30°C for 30 min and then immediately run through a Sephadex G-50 column. DS oligonucleotides were treated with MMS after the annealing process.

OLIGO-NUCLEOTIDE	SEQUENCE: 5′ → 3′	LENGTH (MER)	DESCRIPTION	REF.: SOURCE
I	AACGCTACTACTATTAGTAGAATTGATGCCACC TTTTCAG	40	General DNA sequence	91927.1, ICRF.
II	CTGAAAAGGTGGCATCAATTCTACTAATAGTA GTAGCGTT	40	Complementary sequence to I	91926.1, ICRF
III	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	60	Poly (dA)	107849.2, ICRF
IV	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTT	60	Poly (dT)	107850.2, ICRF
V	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC CCCCCCCCCCCCCCCCCCCCCCCCCC	60	Poly (dC)	108051.1, ICRF
VI	IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIG	60	Poly (dI)	108052.1, ICRF

TABLE 3: OLIGONUCLEOTIDES SYNTHESISED FOR DNA BINDING ASSAYS

OLIGONUCLEOTIDE	DESIGNATION	DESCRIPTION
1	Oligonucleotide I	General DNA sequence (SS)
2	Oligonucleotide II annealed to I	General DNA sequence (DS)
3	Oligonucleotide III	Poly (dA)
4	Oligonucleotide IV	Poly (dT)
5	Oligonucleotide IV annealed to III	Poly (dA)/ poly (dT)
6	Oligonucleotide V	Poly (dC)
7	Oligonucleotide VI annealed to V	Poly (dI)/ poly (dC)

TABLE 4: NOMENCLATURE OF OLIGONUCLEOTIDES FOR DNA GEL SHIFT AND FILTER BINDING ASSAYS

2.13 DNA GEL SHIFT ASSAYS

A 7% non- denaturing polyacrylamide (PAA) gel was made by mixing 40% acrylamide/ bisacrylamide 19:1 (Ultra pure Accugel, gas stabilised, National Diagnostics) with 30% acrylamide/ bisacrylamide 37.5:1 (Page I protein gel mix, Boehringer Mannheim), 0.1% ammonium persulfate, 2.5% glycerol, 0.5x Tris-acetate (TAE) buffer (Maniatis *et al.*, 1982), and N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad).

Reactions were set up by mixing AlkB protein (0 – 90 pmol), 60 pmol bovine serum albumin (BSA, New England Biolabs) or 60 pmol single stranded binding protein (SSB, USB) in enzyme dilution buffer (50 mM Tris HCl pH 7.5, 30 mM NaCl, 1 mM EDTA, 1 mM DTT) with ^{32}P 5' end- labelled SS or DS DNA at 20,000 cpm/ reaction, (Table 4), 0.1 µg/ µl poly (dI)/ poly (dC) a non- specific competitor, 1x binding buffer (25 mM Hepes- KOH pH 7.8, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol) at 4°C for 15 min, and then immediately loaded onto the gel.

The gel was run in 0.5x TAE buffer at 70V, 4°C for 2 h. The gel was dried on a gel dryer (Model 583, Bio-Rad), and exposed overnight to a Molecular Dynamics phosphor screen, and then scanned and quantitated using the Molecular Dynamics Storm 860 machine.

2.14 DNA FILTER BINDING ASSAYS

Varying amounts of protein (AlkB/ SSB, 0 – 15 pmol) in buffer (20 mM Tris HCl pH 7.5, 10% glycerol, 100 mM KCl, 0.1 mM DTT) were incubated with various unmethylated and methylated ^{32}P 5' end- labelled DNA substrates (30,000 cpm/ reaction, Table 4) in a reaction volume of 20 µl at 30°C for 30 min. The reactions were then placed at 0°C. One ml of buffer was added to each reaction, and then directly loaded onto nitrocellulose disc filters (HAW P02500

Scheibenfilter, Millipore) on a vacuum filtration apparatus (Millipore). The filters were washed with 10 ml buffer, and then dried and placed into scintillation vials. 10 ml of scintillation fluid (toluene mixed with Packard permablend III) was added to each vial, and the DNA bound to AlkB on the filter quantitated by scintillation counting (5 min/ sample) using the Beckman LS 6000IC scintillation counter.

To test if AlkB has nuclease activity, 10 pmol AlkB protein was incubated under various conditions (± 5 mM $MgCl_2$/ ± 2 mM manganese chloride ($MnCl_2$)) with ^{32}P 5' end- labelled SS DNA (oligonucleotide 1) in reaction volumes of 20 µl at 30°C, 30 min. At times 0, 5, 15 and 30 min the reaction was terminated by ethanol precipitation. 0.3 M sodium acetate pH 5.2, 10 mM $MgCl_2$, and three volumes 100% cold ethanol were added. The reactions were placed on dry ice for 5 min, then spun at 4°C, 15000 rpm for 15 min. Half the supernatant was taken and placed in a scintillation vial with 5 ml picofluor (Packard). The supernatants were counted (5 min/ sample) for release of soluble counts using the Beckman LS 6000IC scintillation counter.

2.15 MUTAGENESIS EXPERIMENTS

Strains CC101 - CC106 (Cupples and Miller, 1989; Miller, 1992) and CC101 - CC106 *alkB117::Tn3* (SD11 - 16, section 2.6) were grown in minimal media without 0.2% casein acid hydrolysate to A_{450} 0.5. Ten ml of cultures were treated with increasing concentrations of MMS (0 - 47.2 mM (0 - 0.4%)) at 37°C for 20 min. The cells were pelleted by centrifugation at 2200 rpm for 5 min, and the supernatant containing the MMS removed. The pellet was resuspended in 1 ml 1x M9 salts containing 1 mM $MgSO_4$.

To estimate survival, the resuspended cells were serially diluted in 1x M9 salts containing 1 mM $MgSO_4$ and cells plated onto L- agar plates in 3 ml molten LB soft agar. Survival of each strain was monitored by colony counting after

overnight incubation at 37°C.

To estimate mutation frequencies, various dilutions of the resuspended cells were mixed with 3 ml minimal soft agar, (1.75% agar, 1 mM $MgSO_4$, 1x M9 salts, 2 µg/ ml thiamine hydrochloride, 0.2% lactose). Cells were plated onto minimal media plates containing 0.2% lactose instead of glucose, and without 0.2% casein acid hydrolysate (Hy- case amino). Plates were retained at 37°C. Mutation frequencies were estimated after 2 day incubation, by monitoring for the appearance of revertant colonies.

2.16 PREPARATION OF DNA SUBSTRATES FOR *IN VITRO* ASSAYS

2.16.1 PREPARATION OF M13, POLY (dA) AND POLY (dC)

M13 was prepared from *E. coli* strain BS141 (AB1157/ F') as a large-scale preparation as described (Sambrook *et al.*, 1989). Poly (dA) and poly (dC) were obtained from Pharmacia Biotech.

2.16.2 LABELLING DNA SUBSTRATES WITH [^{14}C] MMS

[^{14}C] MMS (1 mCi, a gift from P. Swann, UK) was provided in ether (0.5 mCi/ ml) with a specific activity (SA) of 55 mCi/ mmol. Therefore, the amount of [^{14}C] MMS was 10 µmol/ ml (concentration/ SA), i.e. a concentration of 10 mM.

The MMS was cooled to 4°C. A cooled Hamilton syringe was coated with ether (to prevent hydrolysis of MMS), and used to pipette the MMS. 0.4 mg DNA substrates were treated with 15 mM [^{14}C] MMS, at 25°C for 1 hour. The reactions were stopped by addition of 0.3 M sodium acetate pH 5.2 and three volumes 100% cold ethanol. The reactions were incubated at -80°C, 45 min, centrifuged and washed with 80% ethanol, and then resuspended to the original

volumes in TE pH 8 (10 mM Tris, 1 mM EDTA, laboratory stock). The samples were dialysed (GIB COBRL, dialysis tubing 2.5") overnight at 4°C in 500 ml cold TE pH 8 to remove any remaining [^{14}C] MMS label. DNA concentrations were estimated by A_{260} readings, and counts per minute (cpm) per µl of sample by scintillation counting using the Beckman LS 600IC scintillation counter. Substrates were labelled in the same manner using [^{3}H] DMS, (NEN).

Double stranded substrates were made by annealing complementary strands to the labelled SS DNA strands. Poly (dI) was obtained from Sigma - Aldrich and poly (dT) from Pharmacia Biotech.

2.17 PREPARATION OF CELL EXTRACTS FOR *IN VITRO* ASSAYS

AB1157, BS118 and BS87 cell extracts were prepared by growing the strains in LB medium to A_{600} 0.5. The cells were spun (8k, 10 min), and the pellet washed in PBSA. The pellet was resuspended in extraction buffer (70 mM Tris HCl pH 8, 1 mM EDTA, 1 mM DTT, 5% glycerol) and then sonicated three times, each time for 5 seconds, with 20 second intervals on ice. The samples were spun, and the supernatant taken. The protein concentration of each extract was estimated using Coomassie Protein Assay Reagents (Pierce).

2.18 *IN VITRO* ASSAYS

2.18.1 DNA GLYCOSYLASE ASSAYS

In 100 µl, varying amounts of AlkB protein or crude extracts were added to [^{14}C] MMS treated DNA substrates under different experimental conditions (± 5 mM $MgCl_2$, ± 50 mM NaCl, ± 1 mM EDTA), and incubated at 37°C for 1 hour. The reaction buffer consisted of 0.07 M Hepes- KOH pH 7.8, 0.5 mM EDTA, 1 mM DTT, and 5% glycerol. The reaction was terminated by adding 0.2 mg/ ml calf thymus carrier DNA, 0.3 M sodium acetate pH 5.2, and three volumes

100% cold ethanol, and incubating for 30 min at -80°C. The supernatant was taken without disturbing the pellet, and placed in a scintillation vial with 5 ml picofluor. The supernatants were assayed for release of soluble material by scintillation counting (count 5 min/ sample) using the Beckman LS 6000IC scintillation counter. The pellets left from the assays were washed 2x in 80% ethanol, centrifuging between each wash at 15,000 g, 4°C, 5 min, and then stored at -20°C for later analysis by high pressure liquid chromatography (HPLC).

2.18.2 METHYLTRANSFERASE ASSAYS

The assays were set up as above (2.18.1), except that incubation was for 30 min at 37°C, followed by precipitation. The pellet was resuspended in 0.1M Tris HCl pH 8, and 1 mg/ ml proteinase K added for incubation at 37°C, 2 h. The reaction was then placed on ice and 1% TCA (trichloracetic acid) added to reprecipitate the sample. The sample was put on ice for a further 10 min and the supernatant counted by scintillation counting to assay proteinase K solubilised material.

2.18.3 DIGESTING PELLETS FROM DNA GLYCOSYLASE ASSAYS FOR ANALYSES BY HPLC

Analysis of methylated purines: The pellets from 2.18.1 were resuspended in 90 µl TE buffer. 0.1 M HCl was added and the samples incubated at 95°C, 1 hour to release the adenine and guanine bases. The samples were then dried down to a volume ≤ 20 µl.

Analysis of methylated pyrimidine as deoxycytidine derivatives: The pellets from 2.18.1 were resuspended in distilled water to 4µl. DNaseI (100 units, Sigma- Aldrich), phosphodiesterase I (*Crotalus adamanteus* venom, 0.04 units, Pharmacia Biotech) and *E. coli* alkaline phosphatase (2.5 units, Sigma- Aldrich) were added and the sample incubated overnight at 37°C. The reaction was stopped by adding three volumes 100% cold ethanol, and incubating -80°C, 45

min. The samples were then dried down to a volume ≤ 20 µl.

2.18.4 HPLC ANALYSES

Hydrolysed pellets (section 2.18.3) were analysed via HPLC (Beckman System Gold – Programmable Solvent Module 126), using UV detection (Beckman System gold – Programmable Detector Module 166). Samples were run over a strong cation exchange (SCX, Whatman) column and eluted with a mixed buffer system of degassed 100 mM ammonium formate (NH_3. CH_2O_2) pH 3.2, and methanol (Fischer Scientific International).

For analyses of methylated adenine bases only, a buffer system of 80% NH_3. CH_2O_2, 20% methanol changing over a 30 min period to 60% NH_3. CH_2O_2, 40% methanol with convex gradient curve 3 was used (Beckman Instruments, 1989). For analyses of free methylated adenine and guanine bases together or deoxycytidines, a gradient from 95% NH_3. CH_2O_2, 5% methanol to 60% NH_3. CH_2O_2, 40% methanol changing over a 30 min period with convex gradient curve 3 was used.

Fractions eluted from the column were collected in scintillation vials every 31 seconds for 95 fractions (~ 295 µl volume), and then every minute until fraction 105 (1 ml volume), using a fraction collector (Frac-100, Pharmacia fine Chemicals). Picofluor was added to each fraction, and the samples counted using the Beckman LS 6000IC scintillation counter (count 3 min/ sample). Retention times of peaks observed were compared against standard markers obtained from Sigma- Aldrich.

Chapter 3

CHAPTER 3

Initial Studies

Sensitivity Of E. coli Strains To MMS And $MeOSO_2(CH_2)_2$-Lex In Vivo

Repair Of DNA Strand Breaks In Vivo

This chapter describes initial studies that were carried out to investigate the role of the AlkB protein. Mutant *alkB* cells exposed to alkylation damage show decreased survival (Kataoka *et al.*, 1983), suggesting that AlkB plays a role in repair or recognition of a toxic lesion(s). Potential roles therefore included AlkB involvement in repair of DNA strand breaks, repair of modified bases in DNA, or repair of DNA- DNA or DNA- protein cross- links *in vivo*. These types of damage can all inhibit DNA replication or RNA synthesis, and so are potential toxic lesions.

Sensitivity Of E. coli Strains To MMS And $MeOSO_2(CH_2)_2$-Lex In Vivo

To initially demonstrate the effect of the S_N2 alkylating agent MMS on different *E. coli* strains known to be sensitive to alkylation damage, various *E. coli* strains were streaked across a gradient of MMS in a L- agar plate, and growth across the plate monitored, (Figure 4). The wild type strain AB1157 showed no sensitivity to the MMS at 5.9 mM, and was able to grow across the gradient plate. *E. coli* strains defective in the *alkB* gene product (BS87), or *alkA1* gene product (MS23) both showed sensitivity towards MMS, and were not able to grow across the MMS gradient. The double mutant *tagA1 alkA1* (GC4803) was the most sensitive, and no cell growth was observed.

MMS is known to generate many different types of DNA lesions, and in

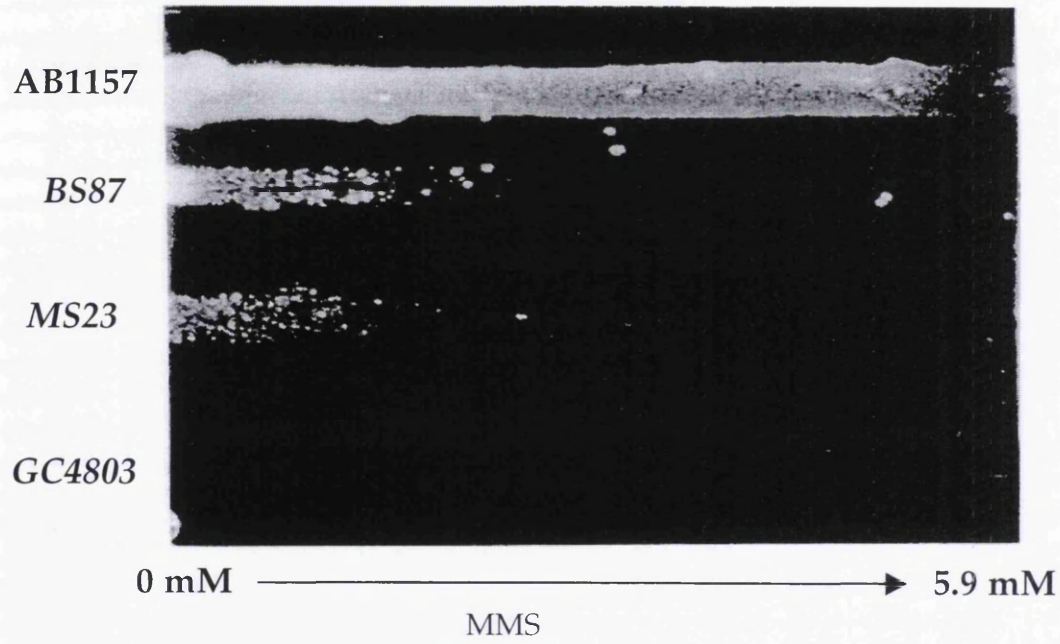


FIGURE 4: SENSITIVITY OF VARIOUS *E. COLI* STRAINS TO MMS

Survival of *Escherichia coli* strains was monitored across a gradient of MMS in a L- agar plate. 10 µl of exponentially growing cells at A_{450} 0.4 in minimal media were streaked onto a L- agar plate of an increasing concentration of MMS (0 - 5.9 mM (0 - 0.05%)). The plate was incubated overnight at 37°C, and then analysed for growth across the plate. AB1157 - wild type; BS87 - *alkB117::Tn3*; MS23 - *alkA1*; GC4803 - *tagA1 alkA1*.

different proportions. We therefore tried to narrow down the possible lesions that *alkB* could be involved in processing. A compound called $MeOSO_2(CH_2)_2$-Lex (Me-Lex) had been synthesised by a group headed by B. Gold, USA. This compound, a methyl sulfonate ester attached to a N'-methylpyrrole dipeptide (Lex) has been designed to specifically target A/T rich regions in the minor groove of DNA to primarily produce the lesion 3MeA (Zhang *et al.*, 1993). Other lesions generated by $MeOSO_2(CH_2)_2$-Lex are produced in very low amounts compared to 3MeA, (Table 5). By monitoring cell killing of various *E. coli* strains by MMS and Me-Lex, we asked if AlkB could be involved in processing the toxic lesion 3MeA that is known to block DNA replication. The wild type strain (AB1157), *alkB* (BS87) and *alkA* (MS23) single mutants were not as sensitive to the Me-Lex, compared to the double mutant *tagA1 alkA1* (GC4803) and triple mutant *tagA1 alkA1 alkB* (SD1) strains, (Figure 5A). The single mutants *alkB* and *alkA1* behaved exactly as the wild type strain. The *tagA1 alkA1* double mutant showed sensitivity towards Me-Lex, with up to a thousand- fold decrease in survival compared to the wild type and single mutant strains at 0.1 mM Me-Lex. The survival of the triple mutant was identical to survival of the double mutant strain.

Survival of the same *E. coli* strains were compared directly to the affects of an increasing dose of MMS, (Figure 5B). The single mutants *alkA1* and *alkB* were sensitive to MMS compared to the wild type parent strain, but less sensitive than the double mutant. This result agreed with the MMS gradient plate result in Figure 4. The striking difference with the compound MMS was the fact that the triple mutant was slightly more sensitive than the double mutant, which was not seen on treatment with Me-Lex. The significance of these observations will be discussed in detail in chapter 8, but the results inferred that AlkB was not involved in processing the toxic lesion 3MeA, but was involved in recognition of another lesion that was produced by MMS, but not by Me-Lex.

MODIFICATION	$MeOSO_2(CH_2)_2$-LEX	MMS
	% Of Total Alkylation	
	Major Groove Lesions	
Adenine N7	nd	(1.8)
Guanine O6 N7	nd 11.7	(0.3) 83
	Minor groove Lesions	
Adenine N3	88	10.4
Guanine N3	0.1	(0.6)
	Other	
Adenine N1	nd	3.8
Cytosine N3	nd	(<1)
Diester	nd	0.8

TABLE 5: NUCLEIC ACID BASE LESIONS INDUCED BY $MeOSO_2(CH_2)_2$-LEX

The different lesions produced by $MeOSO_2(CH_2)_2$-Lex in DS DNA compared to MMS.
nd: derivative not detected; (): estimated value, and so less reliable compared to other data shown.

[Adapted from: Singer & Grunberger, 1983; and personal communication, B. Gold, USA].

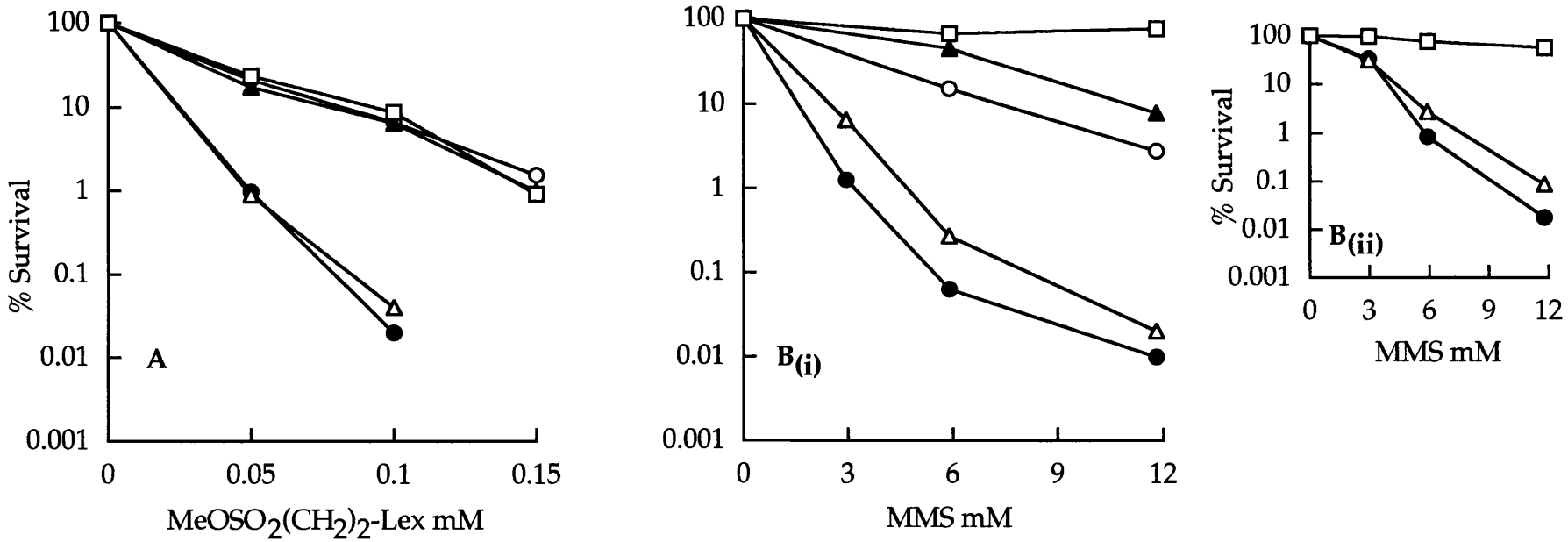


FIGURE 5: SENSITIVITY OF *E. COLI* STRAINS TO $MeOSO_2(CH_2)_2$- Lex AND MMS

E. coli strains at A_{450} 0.5 were treated with an increasing concentration of **(A)** $MeOSO_2(CH_2)_2$-Lex (0 mM - 0.15 mM) for 2 h or **(B)** MMS (0 mM - 11.8 mM) for 20 min, and then assayed for survival.
□: AB1157 (wild type); ▲: BS87 (*alkB117::Tn3*); ○: MS23 (*alkA1*);
Δ:GC4803 (*tagA1 alkA1*); ●: SD1 (*tagA1 alkA1 alkB117::Tn3*)

[$B_{(ii)}$ is a repeat experiment to $B_{(i)}$ to show result reproducibility of the difference between strains GC4803 and SD1].

Another question that was addressed was the ability of AlkB to repair DNA strand breaks *in vivo*. Apart from generating the modified base lesions, alkylating agents have been reported to produce strand breaks (Dolle and Stratling, 1989). Strand breaks were monitored by observing the various forms of the plasmid pET15b. Plasmid DNA was analysed after gentle extraction using the QIAamp tissue kit. This kit was specifically designed to allow DNA extraction avoiding any harsh and denaturing conditions to prevent non-specific cleavage of DNA (Epe *et al.*, 1989; Yakes *et al.*, 1996). The plasmid normally exists in the closed circular (CC) form. If strand breaks were generated by MMS, the plasmid would shift from its closed circular to open circular (OC) form, (Figure 6A). However with an increasing dose of MMS there was no change in the ratio of OC to CC forms of the plasmid pET15b. The *alkB* mutant strain carrying the plasmid (HK82/ pET15b) showed no difference compared to the wild type strain (HK80/ pET15b), (Figure 6B). Analysing survival of the two strains, (Figure 6C), clearly showed that the MMS was having an affect as the *alkB* mutant strain showed a decreased survival with increasing MMS, compared to the wild type. This result suggested that AlkB was not involved in repair of DNA strand breaks, but a different lesion generated by the alkylating agent MMS.

Other studies in our laboratory to identify a function for AlkB have ruled out any potential role in repair of abasic sites *in vivo*, and so any secondary lesions associated with such events such as DNA- protein or DNA - DNA cross- links that could occur (Dinglay *et al.*, 1998).

The data presented here shows that the *alkB* gene product is not involved in repair of the toxic lesion 3MeA, nor of DNA strand breaks *in vivo*. AlkB is sensitive to MMS and probably recognises/ repairs a different lesion generated

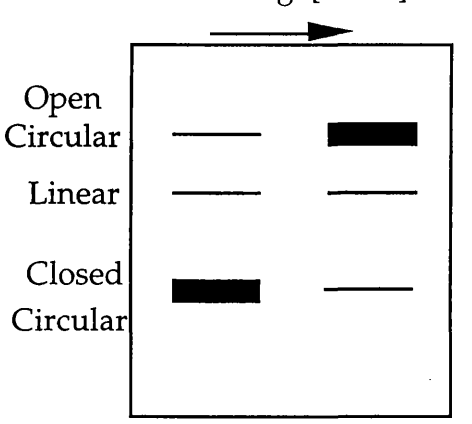
by this compound. Presented in the next chapter are data which changed our direction for future experiments.

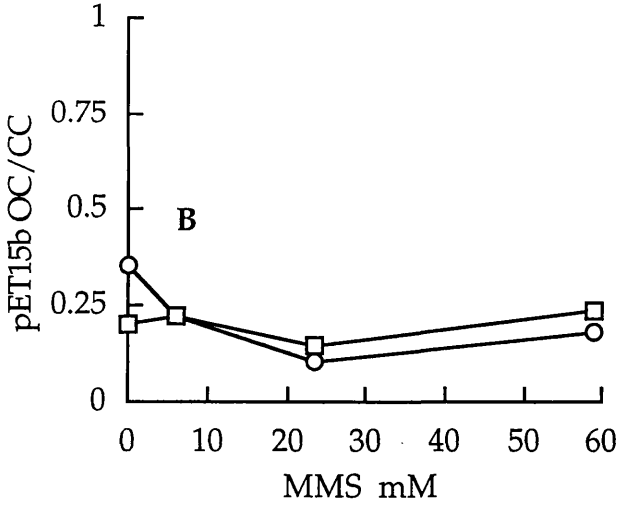
FIGURE 6: REPAIR OF DNA STRAND BREAKS *IN VIVO*

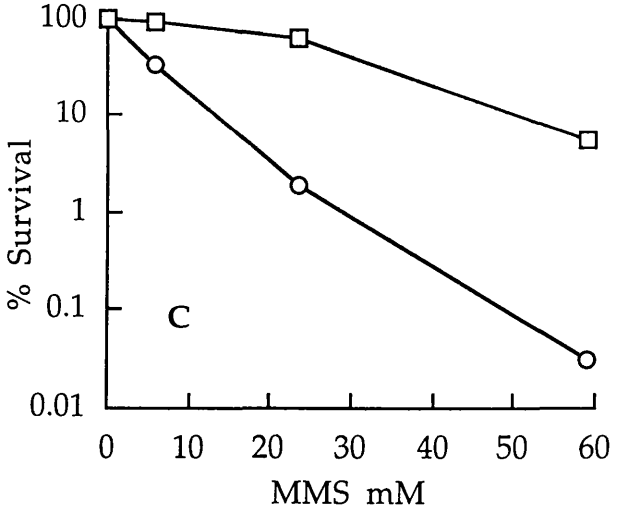
Strains were exposed to various doses of MMS (0 mM (0%) - 59 mM (0.5%)) for 20 min at 37°C. DNA was extracted using the QIAamp Tissue Kit (Qiagen), and analysed for OC and CC forms by gel electrophoresis and scanning of the ethidium bromide gel with a Molecular Dynamics Computing Densitometer. Cell survival analysis was carried out by plating cells on L- agar and monitoring for survival after overnight incubation at 37°C.

(A) Expected result - Plasmids generally exist in the CC form. With increasing doses of MMS, if strand breaks occur, there would be a shift from the CC to the OC form of the plasmid. **(B)** Ratio OC/ CC forms of the plasmid **(C)** Cell survival analysis with increasing concentration of MMS.

□: HK80 (wild type)/ pET15b; ○: HK82 (*alkB22*)/ pET15b







Chapter 4

CHAPTER 4

Processing Of Single Stranded DNA By AlkB

AlkB In Processing Damaged Single And Double Stranded DNA

AlkB And Its Interaction With Other DNA Repair Pathways

Sensitivity Of alkB Mutants To Alkylation Damage In Exponential And Stationary Phase Of Growth

Previous experiments to investigate the possible role of AlkB have used damaged DS DNA as the substrate. Host cell reactivation experiments concluded that *E. coli alkB* mutants were less capable of reactivating MMS treated lambda bacteriophage, with a two- fold decreased capacity compared to the wild type parent (Kataoka and Sekiguchi, 1985). As AlkB is not involved in repair of DNA strand breaks or the toxic lesion 3MeA, (Chapter 3), our attentions were diverted to other lesions generated by alkylating agents, (Table 1). AlkB is sensitive to MMS but less sensitive to MNU (Kataoka *et al.*, 1983), but what is so different between S_N1 and S_N2 agents? Table 1 revealed some striking comparisons. We noted quantitative differences in certain lesions generated by MMS and MNU, and that these amounts were greater in SS DNA. In particular the lesions 1MeA and 3MeC were generated in relatively higher amounts in SS DNA by MMS. No repair protein has been associated with these two lesions. Could AlkB possibly be processing damaged SS DNA? In this chapter we address this question specifically. The possibility of 1MeA and 3MeC as specific targets for AlkB are investigated in DNA binding, mutagenesis and *in vitro* DNA repair assays in the chapters that follow.

AlkB In Processing Damaged Single And Double Stranded DNA

Depicted in Figure 7 is a dramatic defect in the ability of *alkB* mutant cells to process damaged SS DNA, (Figure 7, data from B. Sedgwick). M13, a SS DNA bacteriophage, was treated with MMS and then used to infect wild type and

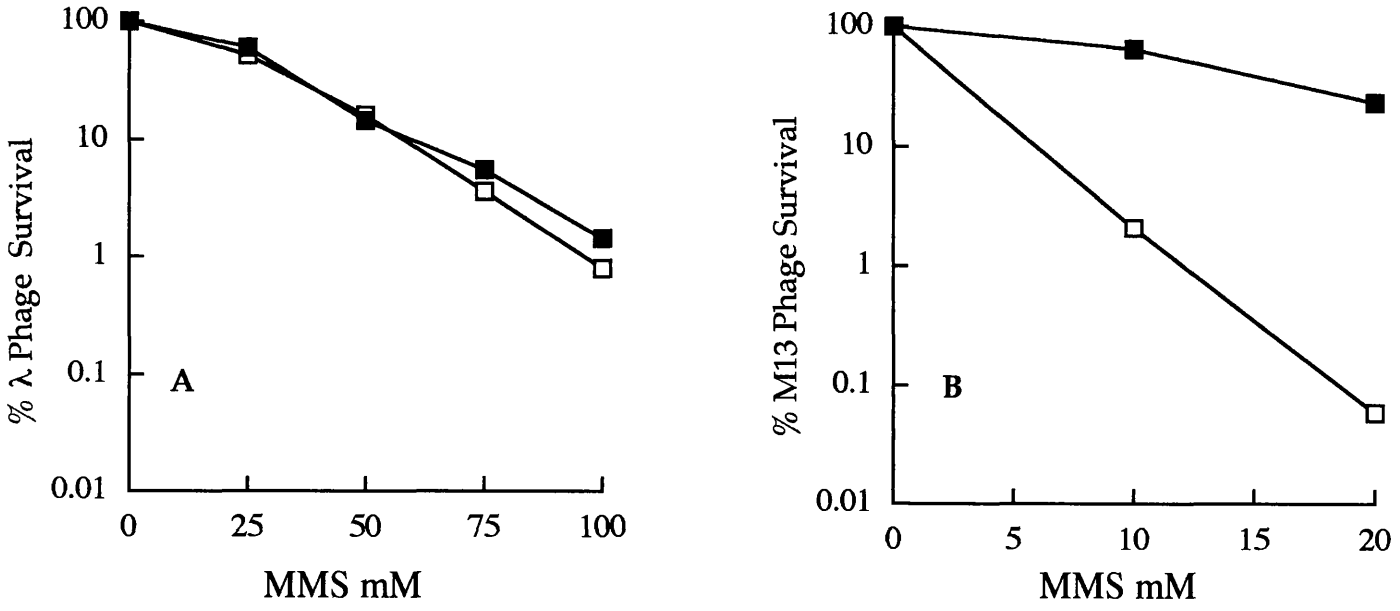


FIGURE 7: DEFECTIVE PROCESSING OF DAMAGED SS DNA IN *alkB* MUTANTS

Defective host cell reactivation of a MMS treated SS DNA bacteriophage (M13) was compared to a DS DNA bacteriophage (λ).

(A) λ was treated with increasing MMS (0 -100 mM) for 30 min at 37°C, and infected into wild type and *alkB* cells. Phage survival was monitored after overnight incubation at 37 °C on L- agar plates. ■: AB1157 (wild type); □: BS87 (*alkB117* ::Tn3)

(B) M13 was similarly treated with MMS (0 - 20mM) for 30 min at 30 °C, and infected into wildtype/ F' and *alkB* / F' cells. Phage survival was monitored after overnight incubation at 37 °C on L- agar plates. ■: BS141 (AB1157/ F'); □: BS143 (BS87/ F')

[*Adapted from data obtained by B. Sedgwick*].

alkB mutant strains. As a control the same experiment was done with a DS DNA λ bacteriophage, a repeat of the earlier experiment done by Prof. Sekiguchi's group (Kataoka and Sekiguchi, 1985). At 20 mM MMS there was a thousand- fold decrease of M13 survival in the *alkB* mutant compared with the wild type, (Figure 7B). This affect was not seen on treatment of λ DNA, (Figure 7A). The result was specific to *alkB* mutants as decreased reactivation of M13 did not occur with *alkA*, or *alkA tag* mutants. Also a *Δ(ada alkB) alkA tag* mutant showed a defect in reactivation of the methylated SS DNA phage as the single *alkB* mutant alone, (data not shown). These findings provide a major step forward towards understanding the role for AlkB as they clearly show that it is involved in processing damaged SS DNA.

The sensitivity of *alkB* mutants as mentioned is specific to damage generated by the S_N2 class of alkylating agents such as MMS. Would this also be true for the reactivation of damaged SS bacteriophage DNA?

M13 bacteriophage was treated with different DNA damaging agents and then used to infect *E. coli* strains. Methyl iodide (MeI) another S_N2 agent, MNU a S_N1 agent, and gamma irradiation known to cause base damage and strand breaks were tested, (Figure 8). Defective processing in *alkB* mutants occurred when M13 was treated with MeI, (Figure 8A), but not with MNU, (Figure 8B), or γ- rays, (Figure 8C). Decreased reactivation was seen with another S_N2 agent DMS, (data not shown). These results confirmed that defective processing of damaged SS DNA in *alkB* mutants was specific to lesions induced by the S_N2 class of alkylating agents.

AlkB And Its Interaction With Other DNA Repair Pathways

AlkB appears to function independently in repair of alkylated DNA (Chen *et al.*, 1994). Would this hold true for processing damaged SS DNA, or would the

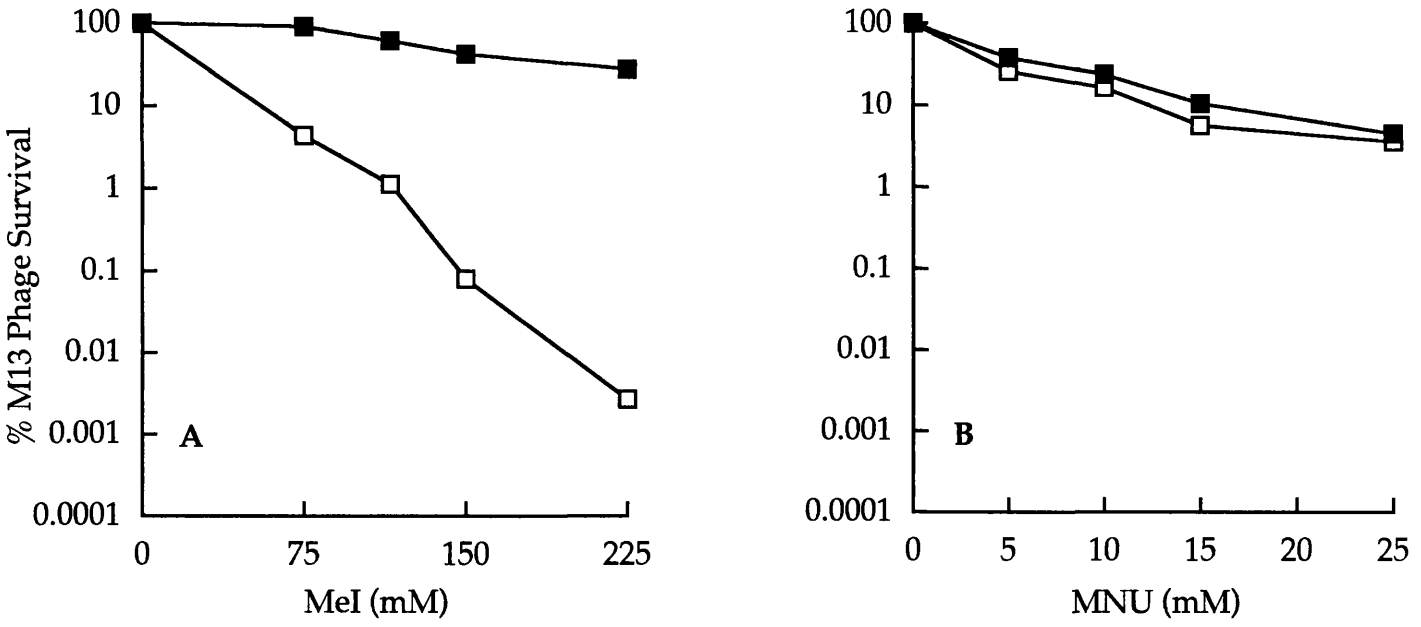


FIGURE 8: ALKB PROCESSES DAMAGE CAUSED BY S_N2 ALKYLATING AGENTS

M13 phage were treated with various DNA damaging agents at 30 °C or 37°C for 30 min, and then infected into wild type/ F' and *alkB* / F' cells. Phage survival was monitored after overnight incubation at 37 °C on L- agar plates.

(A) S_N2 agent MeI: 0 - 225 mM; **(B)** S_N1 agent MNU: 0 - 25 mM; **(C)** γ - Irradiation: 0 - 500 gray.

■: BS141 (AB1157/F'); □: BS143 (BS87/F')

% M13 Phage Survival
100
10
1
0.1
0.01
0.001
0.0001
C
0
100
200
300
400
500
γ-Irradiation (Gray)

alkB gene product need to interact with other known gene products of the DNA repair pathways? To allow infection by M13, F' derivatives of various mutants defective in DNA repair were constructed to allow M13 treatment with MMS, followed by infection of the various *E. coli* strains, (Chapter 2, Section 2.8).

AlkB acted independently of the known DNA repair pathways, (Figure 9). Error prone bypass was not involved as the *umuDC* mutant was not deficient in processing alkylation damage in SS DNA. The double mutant *umuDC alkB* was as deficient as the *alkB* single mutant, indicating that the defective repair was due to the *alkB* gene product alone. Also bypass of the lesion followed by subsequent excision by nucleotide excision repair (NER) was not occurring as interaction with the *uvrA* gene product proved negative, (Figure 9A). Interaction with mismatch repair was investigated too. In the absence of AlkB could a DNA base be incorporated at the site of damage to result in a mismatch? Mismatch repair would be activated to remove the mismatched base, but repeated attempts to do so would result in cell toxicity, analogous to DNA methylation tolerance observed in humans, (Chapter 1, section 1.3). This was not the case as the *mutS alkB* double mutant did not show enhanced survival, and was just as deficient in processing damage as the *alkB* mutant alone, (Figure 9B). We did however observe a slight RecA functional back up. The *recA alkB* double mutant showed a slight increase in defective processing of SS DNA damage in comparison to the *alkB* single mutant. The *recA* and *alkB* mutant deficiencies were additive, indicating that the two genes worked independently. RecA participates in many of the DNA repair pathways, so it is possible it could backup AlkB function too, (Figure 9C).

Sensitivity Of alkB Mutants To Alkylation Damage In Exponential And Stationary Phase Of Growth

During exponential phase of growth the cells are actively replicating and contain more DNA replication forks, and so more regions of SS DNA

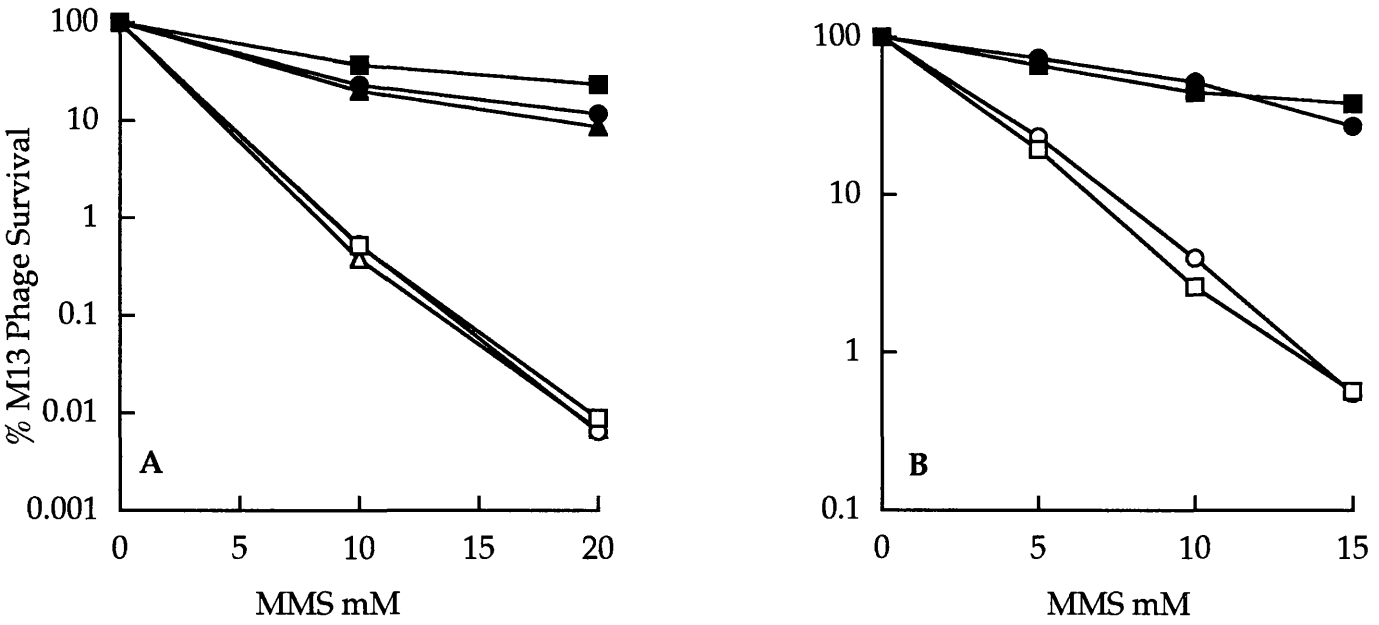
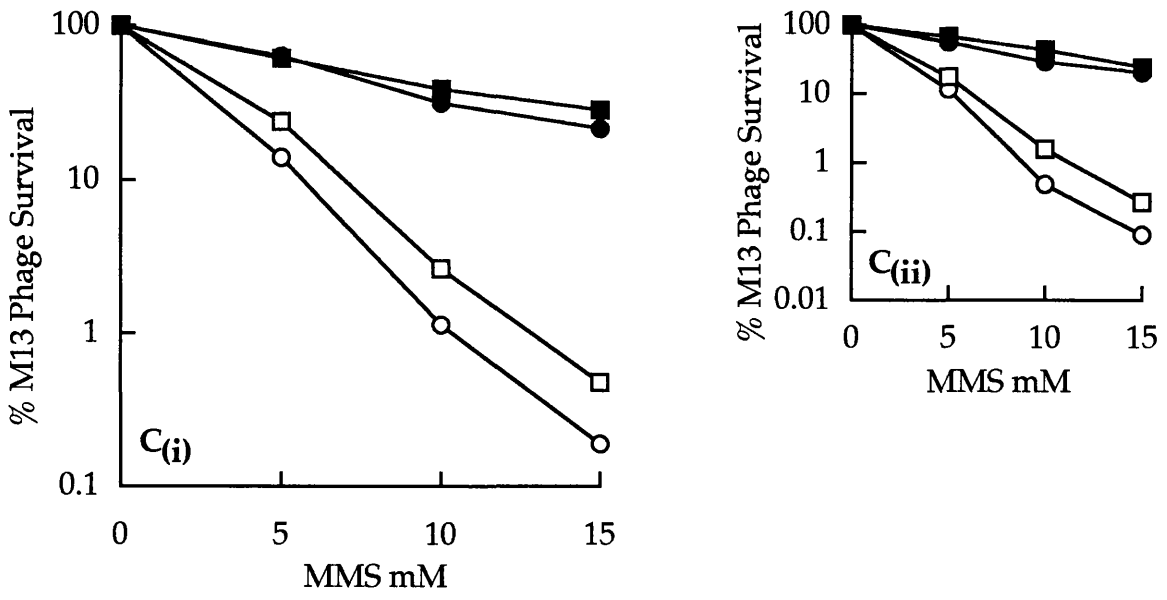


FIGURE 9: ALKB ACTS INDEPENDENTLY OF OTHER KNOWN DNA REPAIR PATHWAYS

M13 phage was treated with increasing MMS (0 - 20mM), 30°C, 30 min, and then infected into various F' strains. M13 phage survival was monitored after overnight incubation at 37 °C on L- agar plates.

(A) *Independent of nucleotide excision and SOS repair.*
■ BS141 (AB1157/ F') - WT/ F'; □: BS143 (BS87/ F') - *alkB* / F'; ●: BS154 (BS146/ F') - *umuDC*/ F';
○: BS155 (BS147/ F') - *umuDC alkB* / F'; ▲: BS152 (AB1886/ F') - *uvrA6* / F'; Δ: BS153 (BS145/ F') - *uvrA6 alkB* / F'.

(B) *Independent of mismatch repair.*
■: BS141 (AB1157/ F') - WT/ F'; □: BS143 (BS87/ F') - *alkB* / F';
●: SD6 (BS163/ F') - *mutS*/ F'; ○: SD7 (BS164 / F') - *mutS alkB* / F'.



(C) *Independent of RecA.*
■: BS141 (AB1157/ F') - WT/ F'; □: BS143 (BS87/ F') - *alkB* / F';
●: SD8 (RW202/ F') - *recA* / F'; ○: SD9 (SD4/ F') - *recA alkB* / F'.

[$C_{(ii)}$ is a repeat experiment to $C_{(i)}$ to show result reproducibility of the difference between strains BS143 and SD9].

[*Figure A adapted from data of B. Sedgwick*].

compared to cells in stationary phase. AlkB processes damaged SS DNA, therefore *alkB* mutant cells may be more sensitive to MMS during exponential growth. Cells in exponential phase of growth were treated with MMS and compared to those in stationary phase, (Figure 10). Wild type cells in both growth phases were not sensitive to MMS at the doses used. *E. coli alkB* mutant cells in exponential phase of growth were more sensitive to MMS than cells in stationary phase, (Figure 10A). It could be argued that cells in stationary phase were not as permeable to MMS, and not as much damage was received by the cells, and so the intermediate sensitivity observed. Therefore the experiment was repeated with an extended MMS dose range until killing in the wild type occurred. At any given concentration the difference in sensitivities of exponential to stationary phase of growth was always much less for wild type compared to the *alkB* mutants, (Figure 10B). Thus the increased sensitivity in exponential phase of growth of *alkB* mutants was probably a result of the increased amount of SS DNA in the cells.

This chapter shows data relevant towards identifying a potential function for AlkB. AlkB processes damaged SS DNA. This activity appears to be specific to the *alkB* gene product. AlkB processes damage generated by the S_N2 agents which include MMS, DMS, and MeI. Its action does not require other gene products from known DNA repair pathways, but a slight RecA functional backup has been implicated.

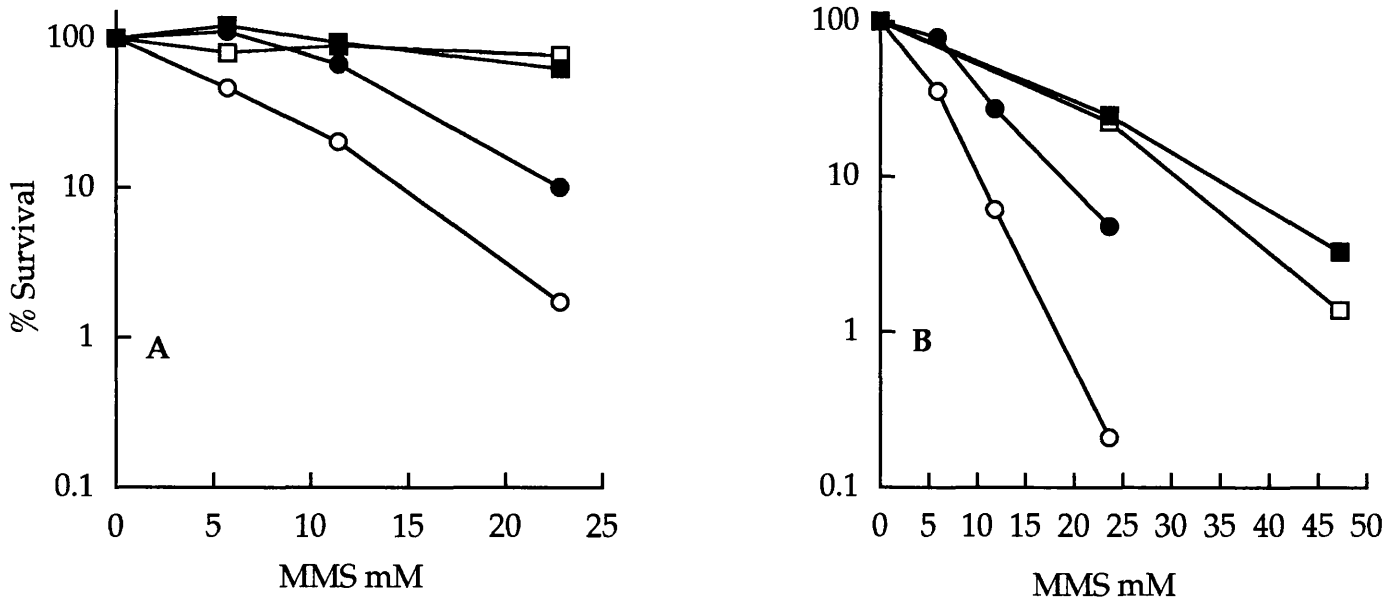


FIGURE 10: SENSITIVITY OF EXPONENTIAL AND STATIONARY PHASE *E. COLI alkB* CELLS TO MMS

E. coli exponential and stationary phase cells were treated with increasing concentrations of MMS for 20 min at 37°C (A), and repeated using an extended range of MMS concentrations (B). Survival was monitored after overnight incubation at 37°C on L- agar plates.

■: AB1157 Stationary (wild type); □: AB1157 Exponential (wild type)
●: BS87 Stationary (*alkB117* ::Tn3); ○: BS87 Exponential (*alkB117* ::Tn3)

Correction - Figure A:
□: AB1157 Stationary (wild type); ■: AB1157 Exponential (wild type)

Chapter 5

CHAPTER 5

DNA Binding Assays

Binding To Single And Double Stranded DNA Cellulose

Gel Shift Assays

Filter Binding Assays

Three different standard binding techniques were set up to investigate whether AlkB binds to DNA. AlkB binding to DNA would further support the evidence that AlkB has a role in DNA processing, even if just in recognition of damage. The affinity of AlkB binding to SS, DS and damaged DNA were compared. Various methylated substrates were also investigated to see if AlkB had an affinity towards a particular type of damage. The ability of AlkB protein to bind to DNA has never been reported, though Dr. Y. Nakabeppu has personally communicated that AlkB binds DS DNA.

Binding To Single And Double Stranded DNA Cellulose

The first binding technique to see if AlkB recognised DNA used single and double stranded DNA cellulose. Purified AlkB was added to a suspension of DNA cellulose, and time allowed for binding to occur. The binding capacity of the DNA cellulose was not limiting. Having a binding capacity of 10 μg protein/ μl DNA, and using a DNA cellulose bed of 30 μl meant a maximum of 300 μg protein capacity, and only 3 μg AlkB was loaded. The DNA cellulose were washed with increasing salt concentrations to elute any bound AlkB. The stronger the affinity of the protein for the DNA cellulose, the higher the salt concentration that would be required to elute off the protein. AlkB was monitored by passing the eluates through a nitrocellulose filter, and probing with polyclonal antibodies raised against AlkB. The secondary antibody was conjugated to horseradish peroxidase. Addition of 4-chloro-1-naphthol (4CIN),

a substrate for this enzyme allowed detection of AlkB by producing an insoluble blue product on the nitrocellulose filter. An AlkB titration was carried out to correspond to the μg amounts of protein loaded in each elutant wash (volumes 1 - 5), so that the intensity and hence recovery of each band could be compared directly. DNA cellulose washed in the absence of AlkB showed no blue product, (SS/ DS DNA + 0 μg AlkB), indicating that the reaction was specific to AlkB. The result showed that AlkB was able to bind both SS and DS DNA, (Figure 11). The unbound AlkB was in the prewash fraction. Most of the AlkB eluted off the matrix in the 150 – 300 mM NaCl washes, and this was the same for both the DS and SS DNA cellulose.

Overall this result suggests that AlkB recognises DNA, both DS and SS DNA with an equal binding affinity. A limitation of this method was that only DS and SS DNA, and not DNA damaged substrates could be investigated. Also the binding affinity to SS vs. DS DNA could not be accurately quantitated, as the antibody detection system could have been saturating. Therefore alternative approaches were used to compare the affinity of AlkB towards SS and DS DNA and various types of damage.

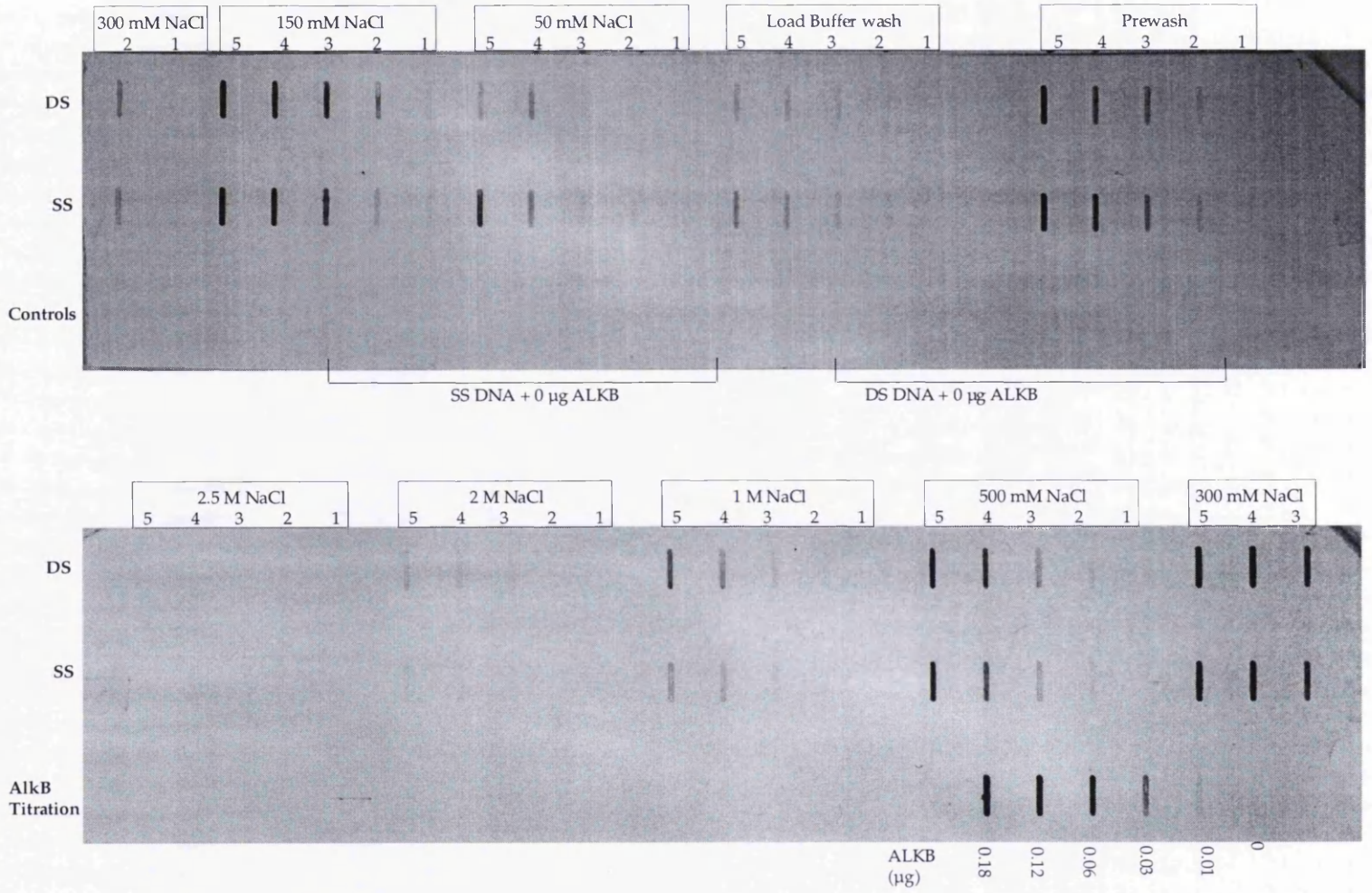
Gel Shift Assays

Two different approaches were used to investigate AlkB/ DNA binding quantitatively. DNA gel shift assays used DNA ^{32}P 5' end- labelled oligonucleotides. The oligonucleotides were incubated with protein and then run through a non- denaturing polyacrylamide gel. Any protein able to bind DNA would retard movement of the DNA fragment through the gel (Ausubel *et al.*, 1999). The bands were quantitated using the Molecular Dynamics Storm 860 machine.

Increasing amounts of AlkB protein (0 – 90 pmol) were incubated with SS or DS oligonucleotides (Table 4, oligonucleotides 1 & 2), and then analysed via

FIGURE 11: BINDING OF PURIFIED ALKB PROTEIN TO SINGLE & DOUBLE STRANDED DNA CELLULOSE

Purified AlkB protein (3 μg) was added to 30 μl suspension bed of single (SS) and double (DS) stranded DNA cellulose, and incubated 0°C, 30 min. The cellulose was then washed with 50 μl of increasing salt concentration (0 - 2.5 M NaCl). Varying volumes of the eluates were loaded onto a Schleicher and Schuell slot blot system. AlkB protein was detected using primary polyclonal AlkB antibody, followed by horseradish peroxidase conjugated secondary antibody.
Titration of eluates (of AlkB protein amount loaded): 1: 0.01 μg; **2:** 0.03 μg; **3:** 0.06 μg; **4:** 0.12 μg; **5:** 0.18 μg.


300 mM NaCl
2 1
150 mM NaCl
5 4 3 2 1
50 mM NaCl
5 4 3 2 1
Load Buffer wash
5 4 3 2 1
Prewash
5 4 3 2 1
DS
SS
Controls
SS DNA + 0 µg ALKB
DS DNA + 0 µg ALKB
2.5 M NaCl
5 4 3 2 1
2 M NaCl
5 4 3 2 1
1 M NaCl
5 4 3 2 1
500 mM NaCl
5 4 3 2 1
300 mM NaCl
5 4 3
DS
SS
AlkB Titration
ALKB (µg)
0.18
0.12
0.06
0.03
0.01
0

non- denaturing polyacrylamide gel electrophoresis (PAGE). An increasing amount of AlkB resulted in an increased intensity of the radioactively labelled bands, and therefore increased binding to DNA, (Figure 12A, lanes 2 - 4 & 6 - 8). Quantitating the bands showed that AlkB was able to bind SS DNA with a greater affinity compared to DS DNA, (~ two- fold difference). This could have reflected a decreased stability of the DS DNA- AlkB protein complex during migration through the PAA gel, (Figure 12B), though the result provided the first evidence that AlkB binds better to SS than DS DNA

Controls for the gel shift assays were set up by analysing the mobility shift of SS and DS DNA by SSB and BSA proteins. SSB was a positive control and bound to both SS and DS DNA, (Figure 13, lanes 3 & 6). It bound SS DNA to a much greater affinity, causing a full shift of the labelled SS oligonucleotide, (Figure 13, lane 3). The BSA was used as a negative control, and as expected was not seen to bind SS or DS DNA, (Figure 13, lanes 2 & 5). These controls showed that DNA binding to AlkB was not an artefact of the technique.

Filter Binding Assays

Although gel shift assays are a very powerful tool in analysing DNA- protein interactions, filter binding assays were used to directly quantitate DNA binding, and did not involve any scanning based methods. Nitrocellulose filters only bind proteins, and therefore labelled DNA bound to protein such as AlkB, is retained on the filters and directly quantified. This approach was not affected by stability of complexes during migration through a PAA gel (Ausubel *et al.*, 1999). The system provided clear and accurate data. AlkB bound to DNA with a greater affinity for SS DNA (Figure 14), which supported the gel shift binding result, (Figure 12). AlkB showed an approximate ten- fold greater affinity towards SS DNA.

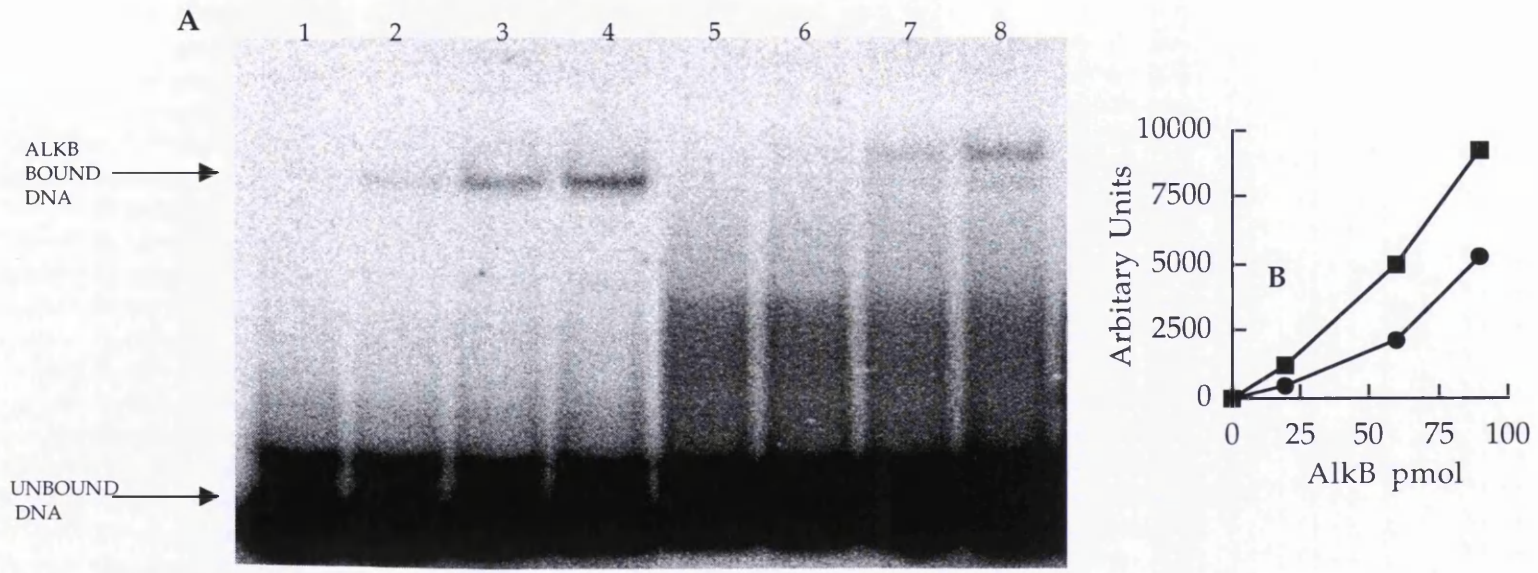


FIGURE 12: GEL SHIFT ASSAY SHOWING ALKB BINDS TO SS AND DS DNA

Increasing amounts of AlkB (0 - 90 pmol) were incubated with ^{32}P 5′ end- labelled SS (oligonucleotide 1) or DS (oligonucleotide 2) DNA at 20,000 cpm/reaction, 4°C, 15 min. The reactions were loaded onto a 7% non- denaturing PAA gel, and electrophoresed at 70V, 4°C, 2 h. The gel was dried and exposed overnight to a Molecular Dynamics phosphor screen. The bands were scanned and quantitated using the Molecular Dynamics Storm 860 machine.

(A) *Gel shift:*

Lanes:	**1:** SS DNA + 0 pmol AlkB	**5:** DS DNA + 0 pmol AlkB
	2: SS DNA + 20 pmol AlkB	**6:** DS DNS + 20 pmol AlkB
	3: SS DNA + 60 pmol AlkB	**7:** DS DNA + 60 pmol AlkB
	4: SS DNA + 90 pmol AlkB	**8:** DS DNA + 90 pmol AlkB

(B) *Quantitating bands from gel shift assay:* ■: SS DNA; ●: DS DNA

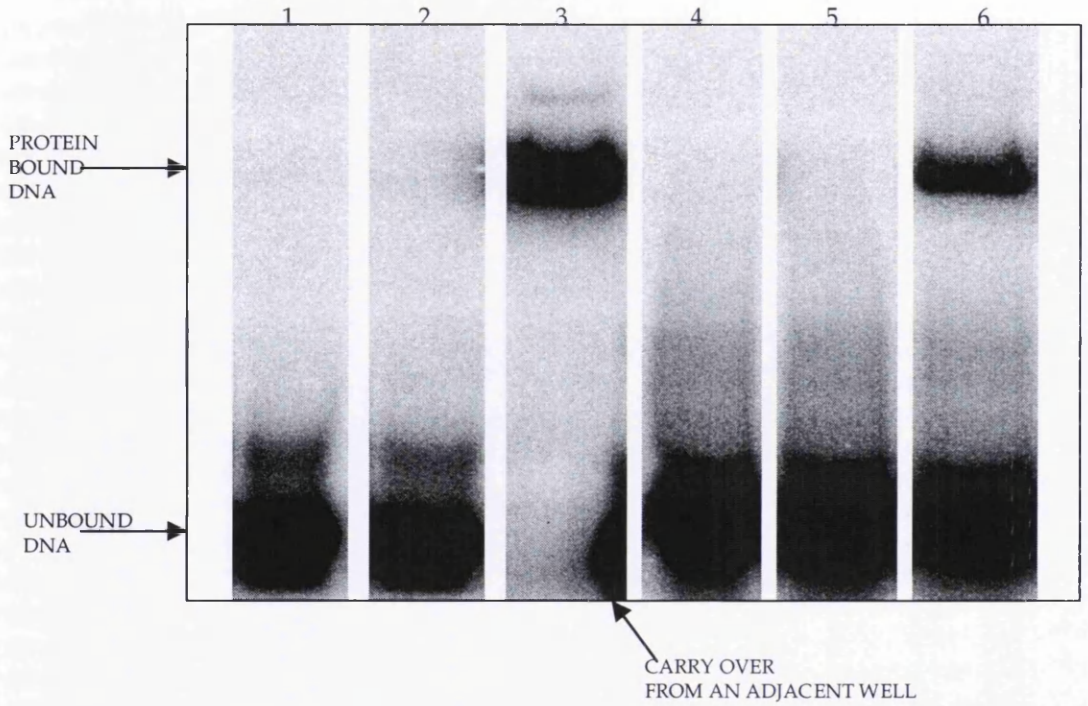


FIGURE 13: CONTROLS FOR GEL SHIFT ASSAY

Single stranded DNA binding protein (SSB - positive control) and bovine serum albumin (BSA - negative control) were set up for the gel shift assay.
60 pmol of BSA or SSB was incubated with ^{32}P 5′ end- labelled SS (oligonucleotide 1) or DS (oligonucleotide 2) DNA at 20,000 cpm/ reaction, 4°C, 15 min. The reactions were loaded onto a 7% non- denaturing PAA gel, and electrophoresed at 70V, 4°C, 2 h. The gel was dried and exposed overnight to a Molecular Dynamics phosphor screen, and then scanned using the Molecular Dynamics Storm 860 machine.

Lanes:
1: SS DNA + 0 protein
2: SS DNA + 60 pmol BSA
3: SS DNA + 60 pmol SSB
4: DS DNA + 0 protein
5: DS DNA + 60 pmol BSA
6: DS DNA + 60 pmol SSB

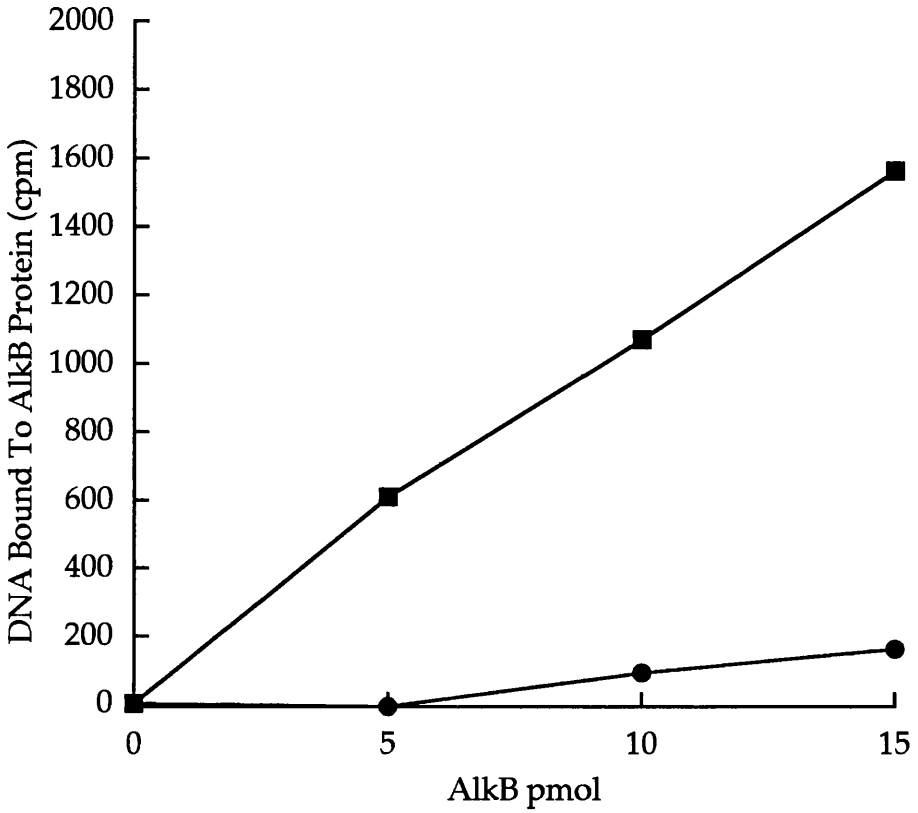


FIGURE 14: BINDING OF ALKB TO SS AND DS DNA

Increasing amounts of AlkB protein (0 - 15 pmol) were incubated with ^{32}P 5' end- labelled SS or DS DNA (30,000 cpm/ reaction), 30°C, 30 min. The reaction mixtures were passed through nitrocellulose filters on a Millipore vacuum filtration system. The DNA bound to AlkB protein retained on the filters was quantitated by scintillation counting.
■: SS DNA (oligonucleotide 1); ●: DS DNA (oligonucleotide 2)

To investigate if AlkB binds damaged DNA to a greater affinity, oligonucleotides 1 and 2, (Table 4) were methylated by treatment with 300 mM MMS. AlkB recognised methylated DNA better (~2- 2.5- fold increase) both in the SS and DS DNA forms, (Figure 15A and B).

Alkylation of DNA by MMS produces many different lesions, and in varying amounts, (Table 1). Upon damage of SS DNA, two lesions produced in significant amounts are 1MeA and 3MeC. We looked at the ability of AlkB protein to recognise these lesions in different damaged substrates. Unmethylated and methylated SS DNA substrates were investigated including oligonucleotides poly (dA), poly (dT) and poly (dC), (oligonucleotides 3, 4 and 6, Table 4). AlkB showed a greater affinity towards pyrimidine based substrates poly (dT) and poly (dC), (Figure 16B and C), compared to the purine based substrate poly (dA), (Figure 16A). The recognition towards methylation still showed approximately two to two and a half- fold increase, whatever the substrate used, as in Figure 15A.

Binding to methylated DS DNA substrates poly (dA)/ poly (dT) and poly (dI)/ poly (dC), (oligonucleotides 5 and 7, Table 4) were also examined, (Figure 17). AlkB bound to these DS DNA substrates with a low affinity, (cf. as in Figure 15B). As expected the methylated DS DNA substrates bound more AlkB. DS methylated poly (dA)/ poly (dT), (Figure 17A) and poly (dI)/ poly (dC), (Figure 17B) bound better compared to the general DS methylated oligonucleotide (oligonucleotide 2, Table 4, cp. Figure 15B). The methylated compared to unmethylated poly (dA)/ poly (dT) still showed the two to two and half- fold increase in binding, but the poly (dI)/ poly (dC) showed a greater difference. Annealing of the homopolymers could have resulted in bubble structures i.e. double stranded DNA with single stranded regions, or double stranded DNA with overhanging single stranded tails. This could have been a better substrate for AlkB recognition, resulting in the increased binding affinity.

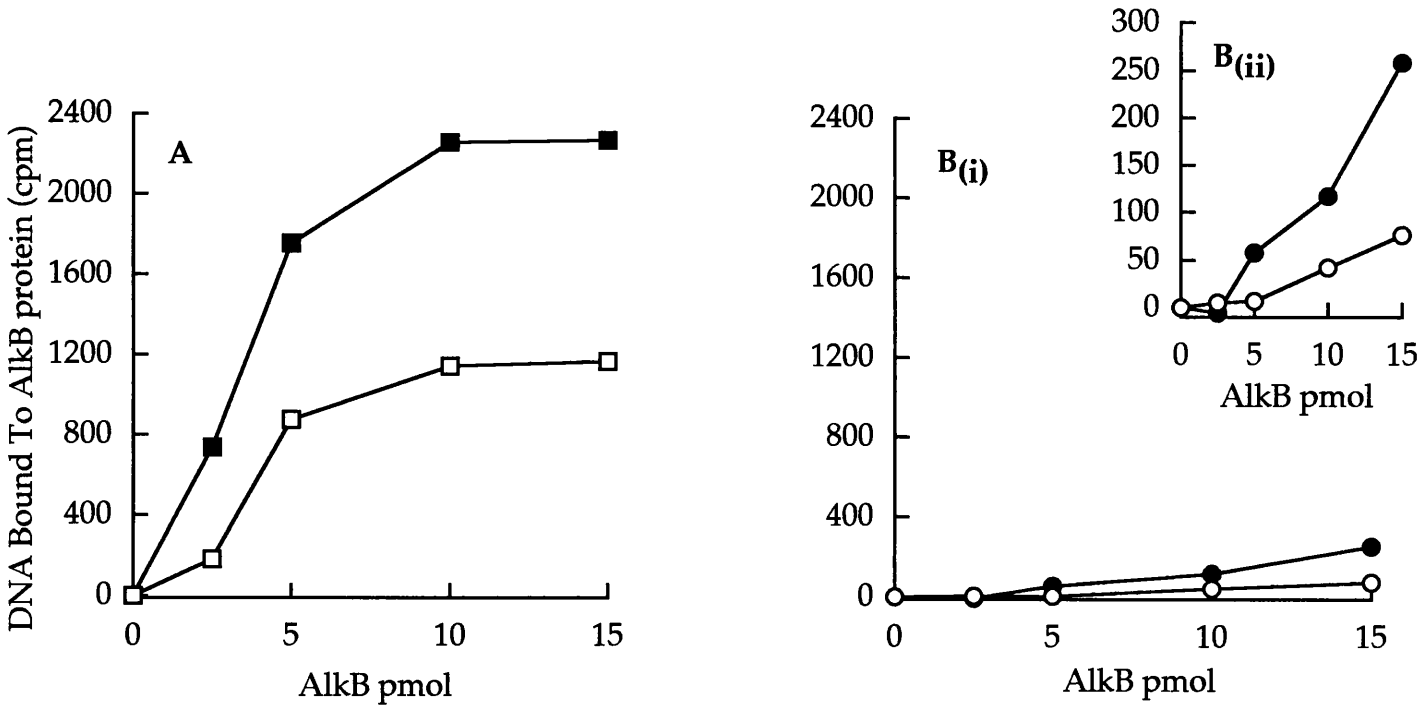


FIGURE 15: BINDING OF ALKB TO UNMETHYLATED AND METHYLATED DNA

Increasing amounts of AlkB protein (0 - 15 pmol) were incubated with unmethylated and methylated ^{32}P 5' end- labelled DNA treated with 300 mM MMS (30,000 cpm/ reaction), 30°C, 30 min. The reaction mixtures were passed through nitrocellulose filters on a Millipore vacuum filtration system. The DNA bound to AlkB protein retained on the filters was quantitated by scintillation counting.

(A) *Single stranded DNA (oligonucleotide 1)* - □: Unmethylated SS DNA; ■: Methylated SS DNA

(B) *Double stranded DNA (oligonucleotide 2)* - ○: Unmethylated DS DNA; ●: Methylated DS DNA

[$B_{(i)}$ and $B_{(ii)}$ show the same data on different scales].

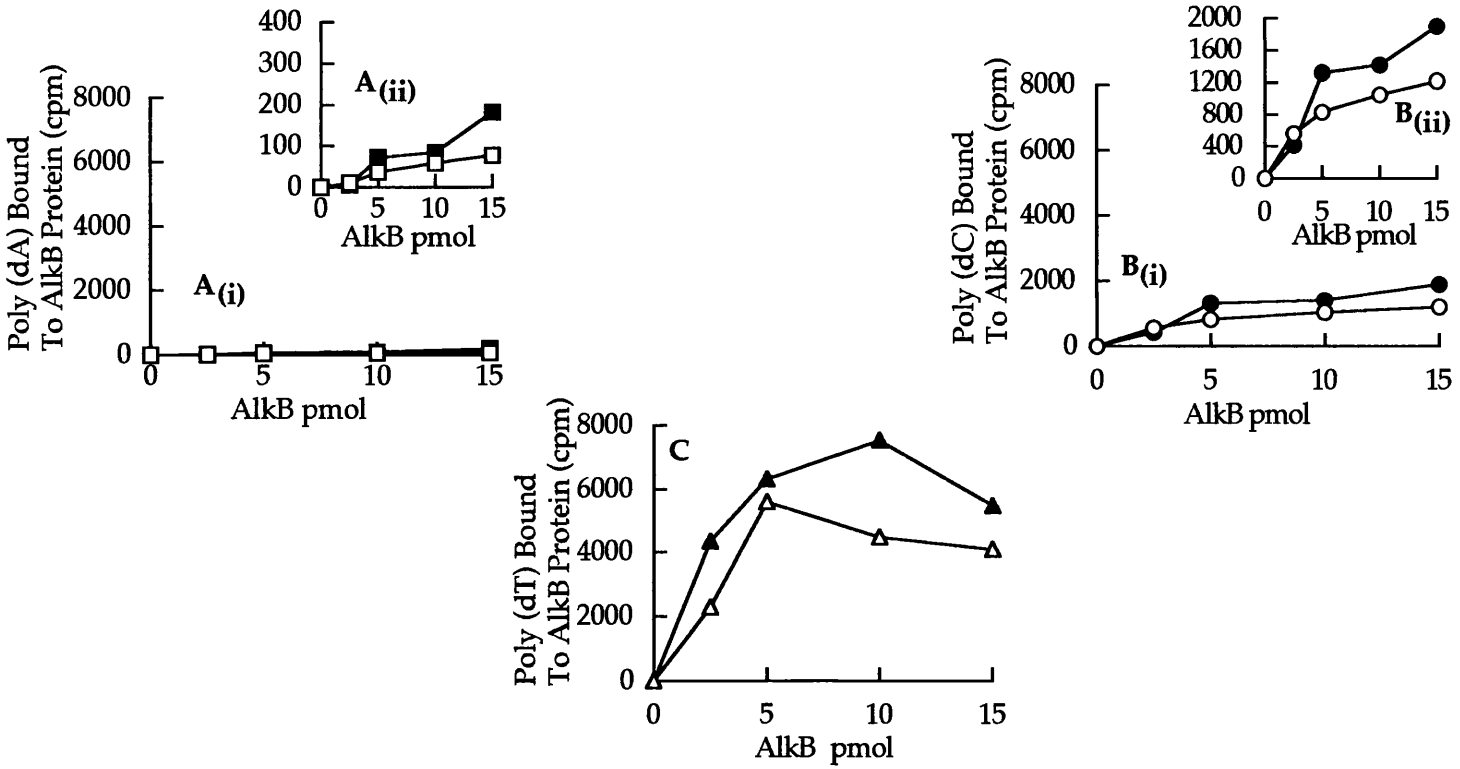


FIGURE 16: BINDING OF ALKB TO SPECIFIC SS DNA SUBSTRATES

Increasing amounts of AlkB protein (0 - 15 pmol) were incubated with specific unmethylated and methylated ^{32}P 5' end- labelled SS DNA substrates treated with 300 mM MMS (30,000 cpm/ reaction), 30 °C, 30 min. The reaction mixtures were passed through nitrocellulose filters on a Millipore vacuum filtration system. The SS DNA substrates bound to AlkB protein retained on the filters were quantitated by scintillation counting.

(A) *Poly (dA) (oligonucleotide 3)* □: Unmethylated Poly (dA); ■: Methylated Poly (dA)
(B) *Poly (dC) (oligonucleotide 6)* ○: Unmethylated Poly (dC); ●: Methylated Poly (dC)
(C) *Poly (dT) (oligonucleotide 4)* Δ: Unmethylated Poly (dT); ▲: Methylated Poly (dT)

[$A_{(i)}$ and $A_{(ii)}$ & $B_{(i)}$ and $B_{(ii)}$ show the same data on different scales].

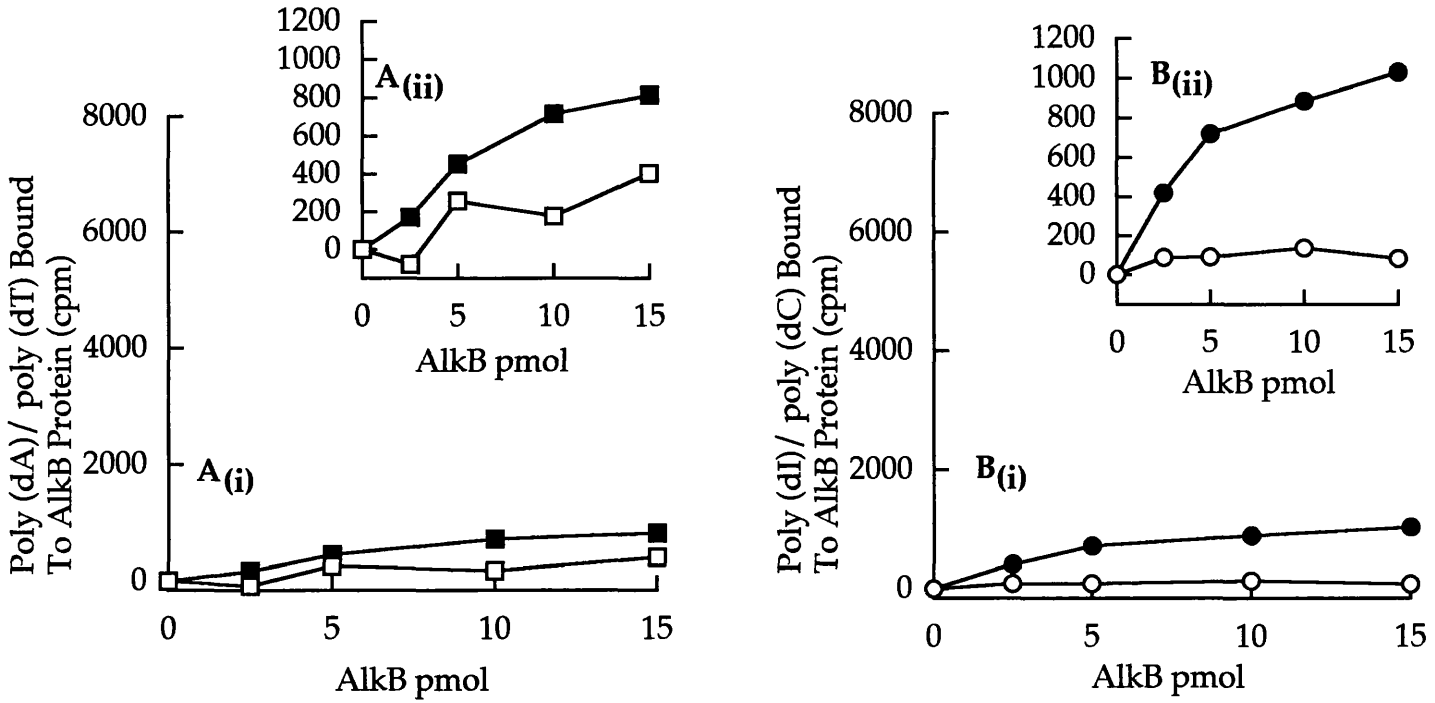


FIGURE 17: BINDING OF ALKB TO SPECIFIC DS DNA SUBSTRATES

Increasing amounts of AlkB protein (0 - 15 pmol) were incubated with specific unmethylated and methylated ^{32}P 5' end-labelled DS DNA substrates treated with 300mM MMS (30,000 cpm/ reaction), 30 ^{o}C, 30 min. The reaction mixtures were passed through nitrocellulose filters on a Millipore vacuum filtration system. The DS DNA substrates bound to AlkB protein retained on the filters were quantitated by scintillation counting.

(A) *Poly (dA)/poly (dT)* *(oligonucleotide 5)* □: Unmethylated Poly (dA)/ poly (dT); ■: Methylated Poly (dA)/poly (dT)

(B) *Poly (dI)/ poly (dC)* *(oligonucleotide 7)* ○: Unmethylated Poly (dI)/ poly (dC); ●: Methylated Poly (dI)/ poly (dC)

[$A_{(i)}$ and $A_{(ii)}$ & $B_{(i)}$ and $B_{(ii)}$ show the same data on different scales].

As a secondary observation in the above experiments, we ruled out any ability of AlkB to act as a nuclease, (Figure 18). AlkB protein was incubated with SS DNA (oligonucleotide 1, Table 4) in the presence or absence of Mg^{2+} and Mn^{2+}, and the release of radioactive soluble material to the supernatant was measured. Generally magnesium ions are often required for nuclease activity, and the release of the counts were seen to slightly increase in the presence of this ion. This was probably attributed to a slight nuclease contamination in the AlkB purification procedure, which is often seen when purifying His- tagged proteins on nickel agarose. If AlkB was a nuclease, we would have excepted a much greater release of counts from the 30, 000 cpm in the initial assay mix, and a complete degradation of the oligonucleotide.

These AlkB/ DNA binding data have shown that AlkB interacts with DNA, and tends to bind SS DNA ± methylation with the greatest affinity. Though compared to SSB, AlkB is a weak DNA binding protein, (Figure 19). Hence, we show here that AlkB is a weak single stranded binding protein, probably recognising damaged SS DNA to a greater extent.

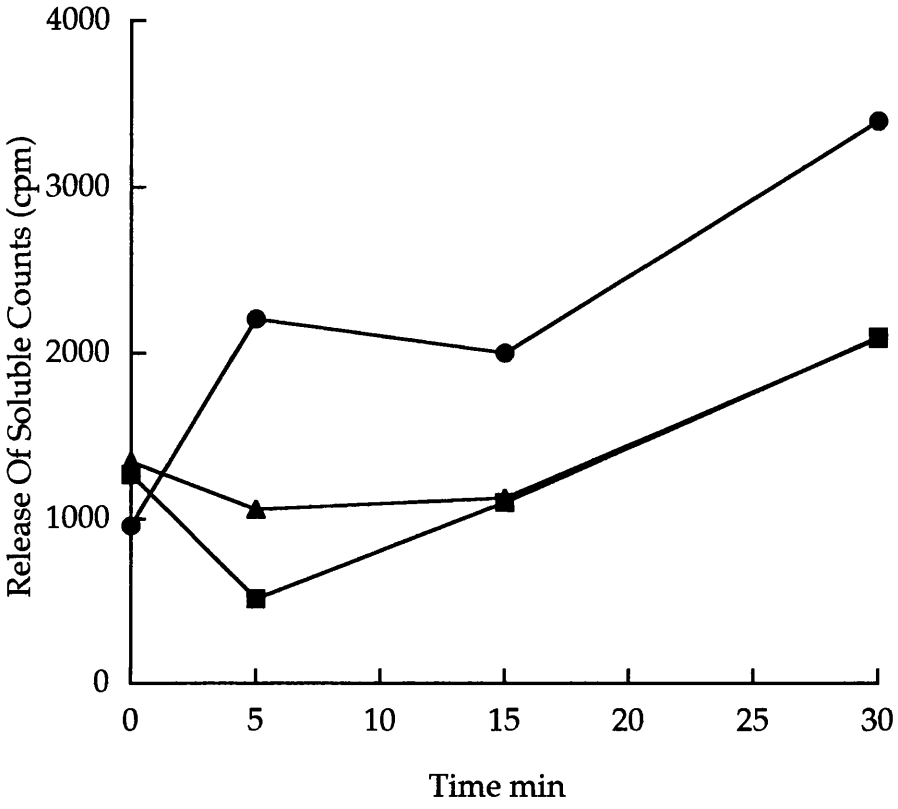


FIGURE 18: ALKB IS NOT A NUCLEASE

A high concentration of AlkB (10 pmol) was incubated with labelled ^{32}P 5' end- labelled SS (oligonucleotide 1) DNA (30,000 cpm/ reaction), under varying conditions at 30°C, over a 30 min period. Samples were taken at 0, 5, 15, and 30 min. The DNA was ethanol precipitated, and each sample was assayed for release of soluble counts to the supernatant.

■: AlkB + SS DNA; ●: AlkB + SS DNA + 5 mM $MgCl_2$; ▲: AlkB + SS DNA + 2 mM $MnCl_2$

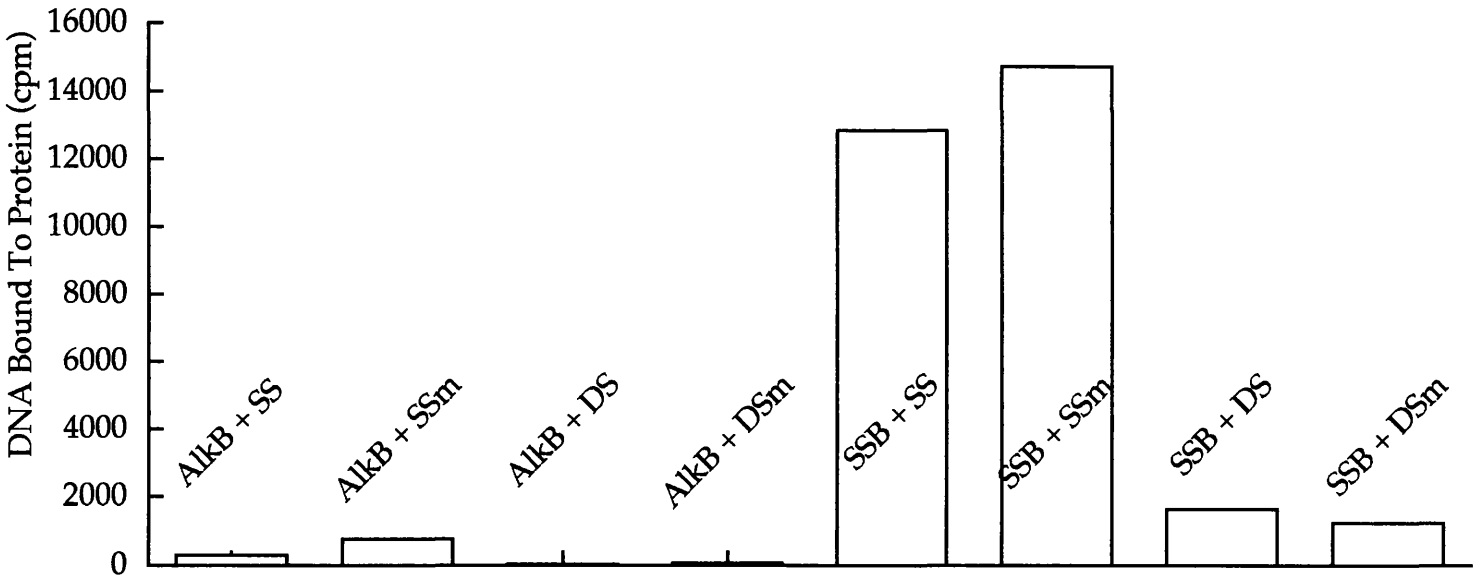


FIGURE 19: ALKB IS A WEAK DNA BINDING PROTEIN

Various proteins (10 pmol) were incubated with unmethylated or methylated ^{32}P 5' end- labelled DNA substrates treated with 300 mM MMS (30,000 cpm/ reaction), 30°C, 30 min. The reaction mixtures were passed through nitrocellulose filters on a Millipore vacuum filtration system. The DNA bound to protein retained on the filters were quantitated by scintillation counting.
SS DNA (oligonucleotide 1); DS DNA (oligonucleotide 2); m - Methylated DNA; SSB - Single stranded DNA binding protein

Chapter 6

CHAPTER 6

Mutagenesis Studies

The previous chapters established that AlkB binds DNA and processes damaged single stranded DNA. Mutagenesis studies in this chapter address two questions as regards AlkB function. Could AlkB itself be involved in error prone bypass by inserting a DNA base opposite a site of damage, its activity giving rise to an increased mutation frequency? 1MeA and 3MeC are produced in high proportions relative to other lesions generated by MMS damage in SS DNA, (Table 1), so maybe activity involves bypass of these particular lesions? Secondly in the absence of AlkB, are lesions bypassed in an error prone manner to ensure continued DNA replication? In this case, we can analyse a spectrum of mutations to help identify the lesion that AlkB may process.

The lactose operon consists of three structurally linked genes *z*, *y* and *a* encoding enzymes for lactose utilisation. The *z* gene product encodes a β-galactosidase enzyme; the *y* gene product encodes for a β-galactoside permease which is a transport protein; and the *a* gene product encodes a thiogalactoside transacetylase (Mathews and van Holde, 1990). β-galactosidase catalyses the breakdown of the carbon source lactose into glucose and galactose. Substitution of glutamic acid (Glu-461) at the active site of this enzyme with any other amino acid lowers enzyme activity and suppresses growth of the *E. coli* strain on a lactose medium (Cupples and Miller, 1988). Six *E. coli* strains each with a chromosomal deletion of the lactose operon, and all carrying a F'*lac* episome with a different point mutation in the Glu-461 codon 'GAG' of the *lacZ* gene have been constructed by a group headed by J. Miller. These resulted in a Lac^- phenotype, that is, inability to grow on a lactose medium. If a base substitution reverts the mutated codons back to GAG, the positive Lac^+ phenotype of the

strain is restored to allow growth on lactose. The six strains are designated CC101 - CC106 (Cupples and Miller, 1989). Due to the different point mutations in each strain, a different base substitution must occur to result in various transversions or transitions to restore the GAG codon and the positive phenotype of the strain, (Table 6).

Mutant *alkB* derivatives of each strain were constructed by P1 transduction and designated CC101 *alkB* - CC106 *alkB*, (Chapter 2, section 2.6). Each strain was treated with an increasing dose of MMS. Survival was monitored on L- agar plates, and induction of Lac^+ mutations were monitored by allowing the strains to grow on a lactose medium. Mutation frequencies were expressed as number of Lac^+ revertants/ 10^6 surviving cells. Would one particular strain show an increased mutation frequency, and give an insight into a particular lesion bypassed?

The *E. coli ada ogt* mutant is O^6MeG methyltransferase deficient, and unrepaired O^6MeG lesions mispair with thymine bases to produce G:C to A:T transitions. An increased mutation frequency is observed in CC102 a*da ogt* on exposure to methylating agents (Mackay *et al.*, 1994). In our studies we used an analogous strain PT11, a CC102 *Δ(ada alkB) ogt* as a positive control. In comparison to the CC102*Δ(ada alkB) ogt* mutant CC101 *alkB* - CC106 *alkB* strains had low frequencies of MMS induced mutations, (Figure 20).

Survival and Lac^+ mutation induction of each strain CC101 - CC106 and their *alkB* derivatives were monitored after treatment with an increasing dose of MMS. All the *alkB* derivatives were sensitive to MMS as expected, (Figure 21 A - F). Low frequencies of Lac^+ revertants of strains CC102 *alkB*, CC104 *alkB* and CC105 *alkB* were detected, (Figure 22A, B and C). Strains CC101 *alkB*, CC103 *alkB* and CC106 *alkB* showed non- detectable mutation frequencies, (Figure 22D). This was not due to a low survival of the strains, as similar

STRAIN	BASE SUBSTITUTION IN GAG CODON OF *LacZ* GENE	AMINO ACID SUBSTITUTED FOR GLUTAMIC ACID	BASE MISPAIRING DETECTED	MUTATIONS DETECTED
CC101	TAG	Amber	T mispairs C or A mispairs G	A.T → C.G transversion
CC102	GGG	Glycine	G mispairs T or C mispairs A	G.C → A.T transition
CC103	CAG	Glutamine	C mispairs C or G mispairs G	G.C → C.G transversion
CC104	GCG	Alanine	C mispairs T or G mispairs A	G.C → T.A transversion
CC105	GTG	Valine	T mispairs T or A mispairs A	A.T → T.A transversion
CC106	AAG	Lysine	A mispairs C or T mispairs G	A.T → G.C transition

TABLE 6: STRAINS USED IN *IN VIVO* MUTAGENESIS WITH BASE CHANGES IN THE *LacZ* GENE

[Adapted from: Cupples & Miller, 1989].

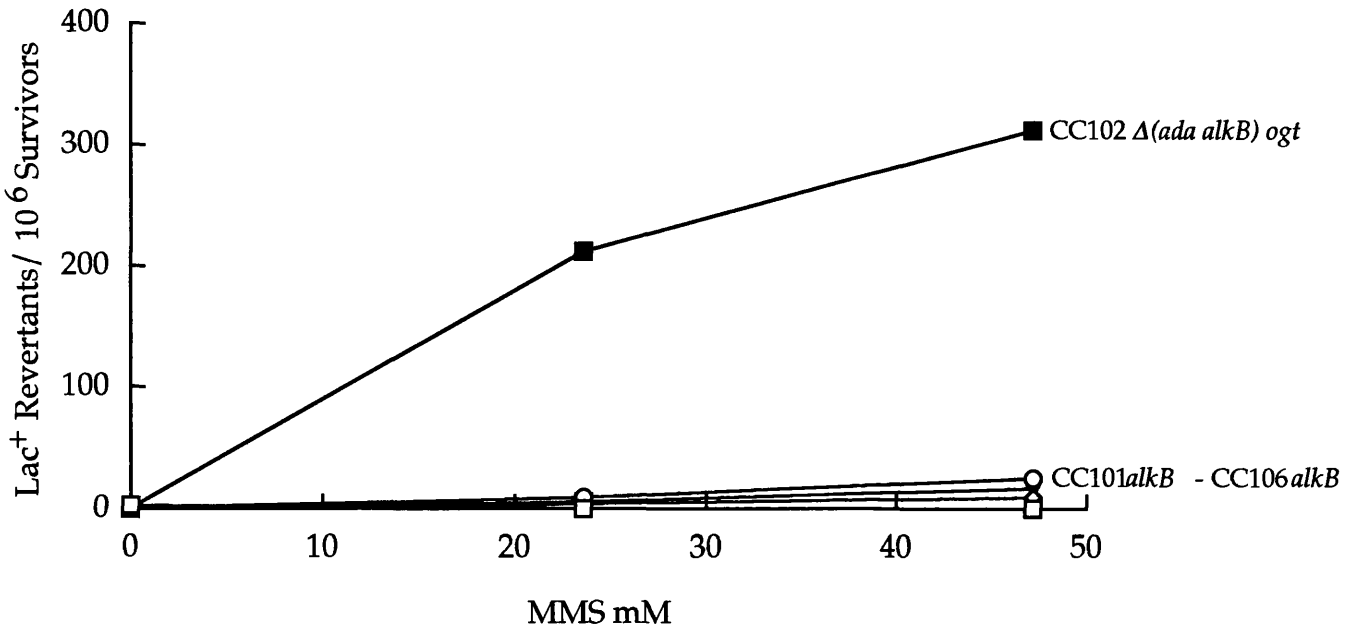


FIGURE 20: CC101 *alkB* - CC106*alkB* MUTANTS SHOW A LOW MUTATION FREQUENCY COMPARED TO A CC102*Δ(ada alkB) ogt* STRAIN

Strains were grown to A_{450} 0.5 and then treated with increasing concentrations of MMS (0 - 47.2 mM), 37°C, 20 min. Lac^+ revertants were monitored after plating on minimal media plates containing lactose, and incubating for 2 days at 37°C.

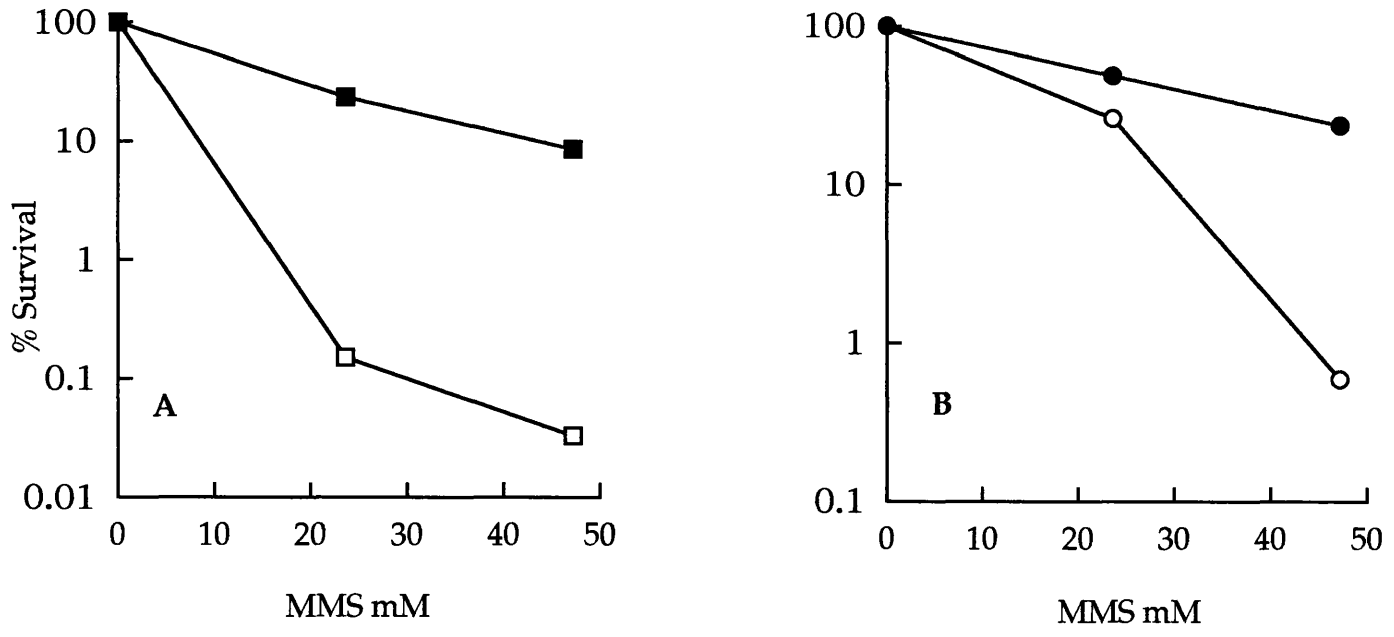


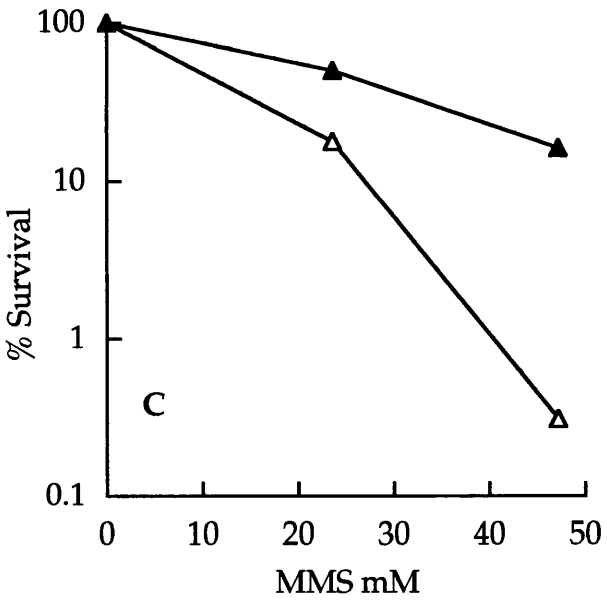
FIGURE 21: MMS SURVIVAL OF CC101 *alkB* - CC106 *alkB* STRAINS

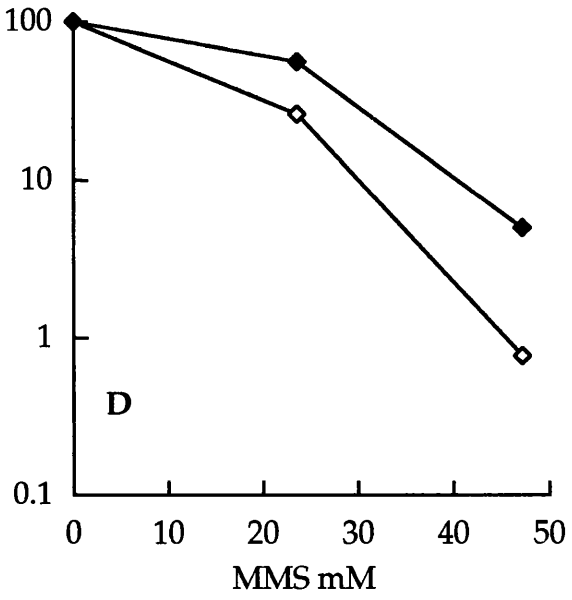
Strains were grown to A_{450} 0.5 and then treated with increasing concentrations of MMS (0 - 47.2 mM), 37°C, 20 min. Survival was monitored after plating on L- agar plates, and incubating overnight at 37 °C.

N.B. Graphs A - F presented are on different scales.

(A) ■: CC101; □: CC101 *alkB* (SD11)

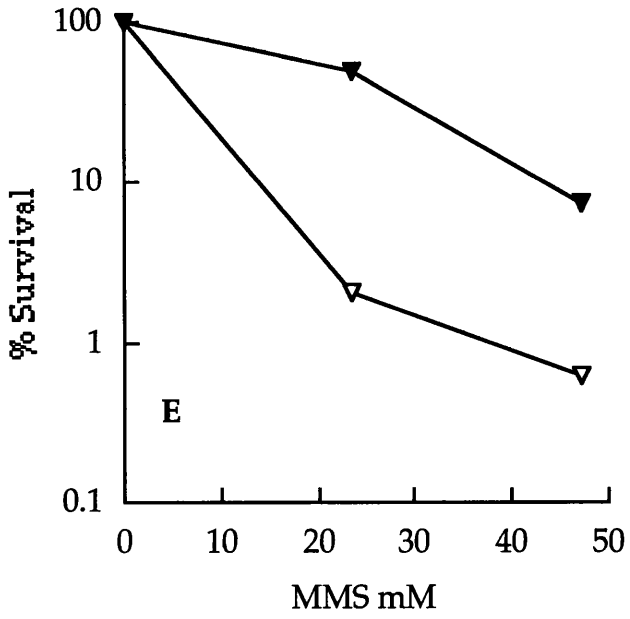
(B) ●: CC102; ○: CC102 *alkB* (SD12)

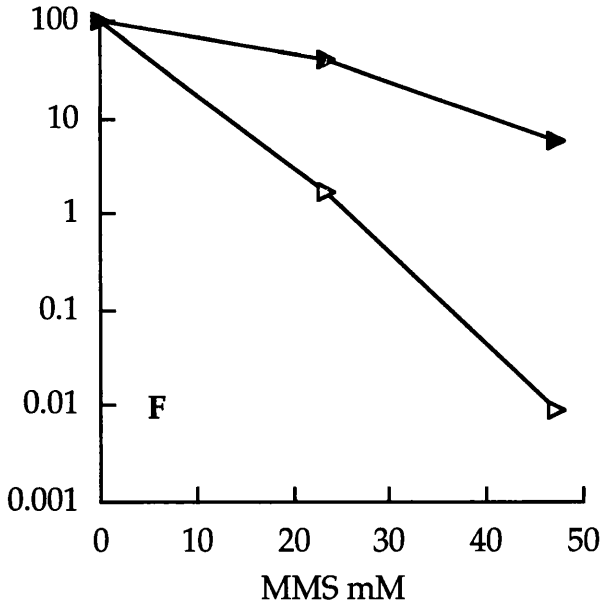




(C) ▲: CC103; Δ: CC103 *alkB* (SD13)

(D) ✦: CC104; ✧: CC104 *alkB* (SD14)





(E) ▼: CC105; ▽: CC105 *alkB* (SD15)

(F) ▶: CC106; ▷: CC106 *alkB* (SD16)

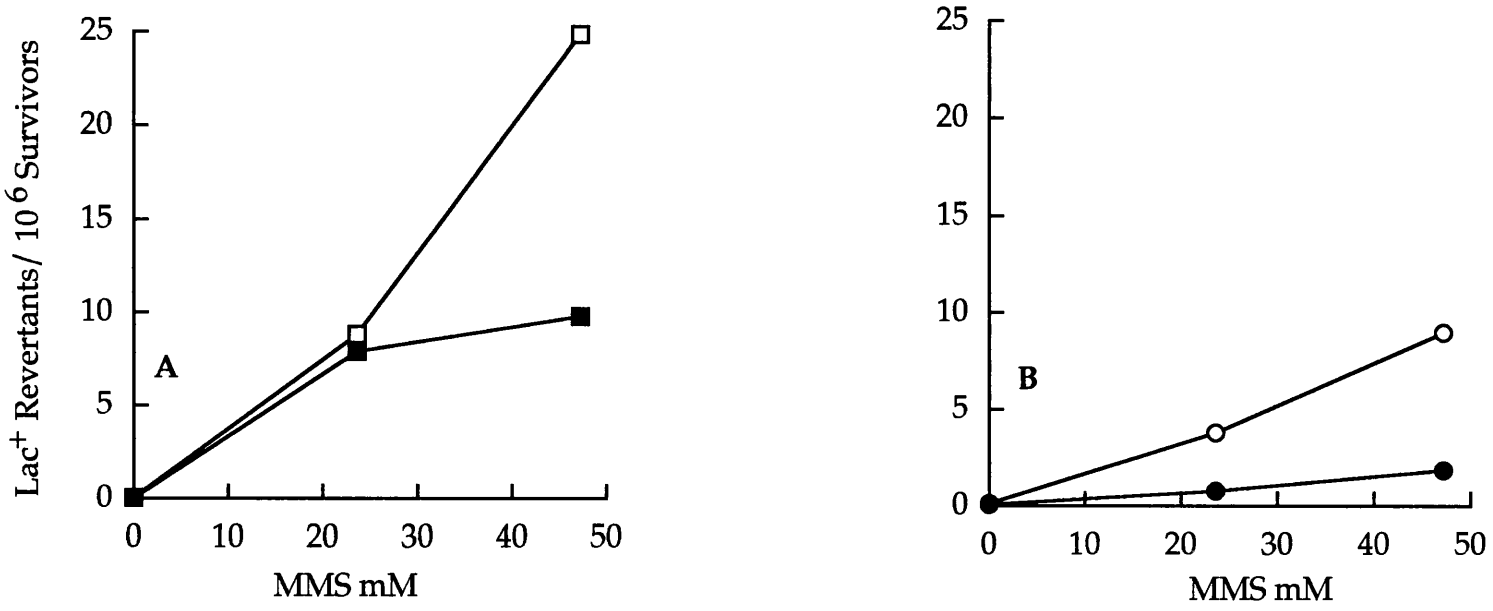
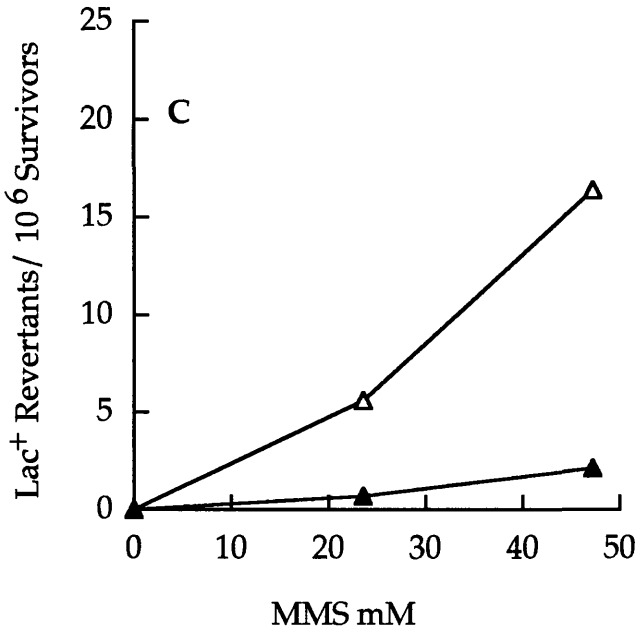


FIGURE 22: MMS MUTAGENESIS OF CC101*alkB* - CC106 *alkB* STRAINS

Strains were grown to A_{450} 0.5 and then treated with increasing concentrations of MMS (0 - 47.2 mM), 37°C, 20 min. Lac^+ revertants were monitored after plating on minimal media plates containing lactose, and incubating for 2 days at 37 °C.

(A) ■: CC102; □: CC102 *alkB* (SD12)

(B) ●: CC104; ○: CC104 *alkB* (SD14)



D

CC101 & CC101*alkB* (SD11)	nd
CC103 & CC103*alkB* (SD13)	nd
CC106 & CC106*alkB* (SD16)	nd

(C) ▲: CC105; Δ: CC105*alkB* (SD15)

(D) nd: non- detectable mutation frequency

survival frequencies in other strains had produced Lac$^+$ revertants. Therefore, if a mutagenic effect was present for CC101 *alkB*, CC103 *alkB* and CC106 *alkB* this would have been detected.

A summary of the Lac$^+$ revertant mutation frequencies are presented in Table 7. The ratio of the MMS induced Lac$^+$ mutation frequencies in the *alkB* strains have been tabulated with respect to those in the wild type. The CC102 *Δ(ada alkB) ogt* mutant shows the greatest increase in Lac$^+$ revertants with increasing MMS dose. Revertant frequencies of CC102 *alkB*, CC104 *alkB* and CC105 *alkB* strains were low in comparison to the CC102 *Δ(ada alkB) ogt* strain. The strains able to revert back to Lac$^+$ all involved a transition or transversion to an adenine base on the sense strand. It is possible that translesional synthesis past a lesion results in incorporation of adenine bases. In the wild type strains when AlkB was present there was no significant mutagenic affect of MMS. It was only in the absence of the *alkB* gene product that the low revertant frequencies above wild type background numbers were observed. These results suggest that AlkB is normally involved in efficient repair/ removal of a lesion, and there is an inefficient error prone bypass mechanism in its absence, resulting in a low MMS induced mutation frequency. Processing of lesions by AlkB itself is therefore not error prone. We had ruled out in chapter 4 any interaction of *alkB* with the *UmuDC* gene product, (Chapter 4, Figure 9A). This will be discussed in more detail in chapter 8.

We have used six strains CC101 - CC106, each carrying a different point mutation in the *lacZ* gene. By transducing *alkB* into these strains and treating with increasing MMS doses, we examined whether any particular strain showed an increased mutation frequency. Even though no particular strain stood out, we were able to show that in the absence of the *alkB* gene product three strains, CC102 *alkB*, CC104 *alkB* and CC105 *alkB* were slightly more sensitive to MMS induced mutagenesis.

STRAIN	BASE SUBSTITUTION IN GAG CODON OF *LacZ* GENE	OBSERVATION	RATIO : *alkB*/ WT MUTATION Lac$^+$ FREQUENCY		
			0 mM MMS	23.6 mM MMS	47.2 mM MMS
CC101 & CC101 *alkB*	TAG	A.T → C.G transversion	126?	nd	nd
CC102 & CC102 *alkB*	GGG	G.C → A.T transition	1.8	1.1	2.5
CC103 & CC103 *alkB*	CAG	G.C → C.G transversion	nd	nd	nd
CC104 & CC104 *alkB*	GCG	G.C → T.A transversion	2.2	5.0	4.9
CC105 & CC105 *alkB*	GTG	A.T → T.A transversion	0.2	8.1	7.7
CC106 & CC106 *alkB*	AAG	A.T → G.C transition	nd	nd	nd
CC102 & CC102 *Δ(ada alkB) ogt*	GGG	G.C → A.T transition	17.6	29.1	

TABLE 7: RATIOS SHOWING *alkB*/ WILD TYPE Lac$^+$ MUTATION FREQUENCIES

Data points for mutation frequencies (revertants/ 10^6 surviving cells) were taken at each MMS concentration for wild type and *alkB*. The ratio was calculated by dividing the *alkB* mutation frequency by the wild type mutation frequency.

nd: non – detectable mutation frequency

Chapter 7

CHAPTER 7

In Vitro Analyses

In vitro DNA glycosylase assays

In vitro methyltransferase assays

In vitro HPLC assays

In this final results chapter the ability of AlkB to process the candidate lesions of interest, 1MeA and 3MeC in DNA, was investigated. Binding experiments showed that AlkB binds methylated DNA especially SS DNA to a greater affinity, and the mutagenesis studies observed no promutagenic affect of AlkB action. Could the role of AlkB be to simply remove the methylated base, or possibly to manipulate the lesion to an alternative form that is harmless? Possible enzymatic activities for AlkB investigated previously included DNA methyltransferase, glycosylase and endonuclease activities (Kataoka and Sekiguchi, 1985). The investigations proved negative but always used damaged DS DNA as the substrates. Shown here are similar investigations using damaged SS DNA substrates.

SS DNA substrates used included M13 SS DNA, poly (dA) for 1MeA analysis and poly (dC) for 3MeC analysis. All were treated either with [^{14}C] MMS or [^{3}H] DMS. Complementary strands were annealed to the treated M13, poly (dA) and poly (dC) substrates to examine whether AlkB recognises damage generated in SS DNA, but processes it only when in DS DNA.

In vitro DNA glycosylase assays

Glycosylase assays determine whether AlkB catalyses the breakage of a glycosyl bond between a damaged base and the DNA sugar- phosphate backbone. Varying amounts of AlkB protein were incubated with the DNA

substrate, and after DNA precipitation release of soluble counts to the supernatant was assayed. The assays were also performed using cell extracts of an *alkB* mutant (BS87) compared to a wild type extract (AB1157). Over-expressing AlkB extracts (BS118) were tested as well. All assays were done under various experimental conditions. A summary table of the assay conditions and results are presented in Table 8. We could not detect that AlkB had any DNA glycosylase activity.

In vitro methyltransferase assays

Methyltransferase activity was tested by incubating AlkB with the various SS DNA substrates to see if it would transfer the methyl group from a lesion to itself. Pellets after the DNA precipitation step which also brings down protein, were treated with proteinase K. Proteinase K digests the protein, and releases amino acids to the supernatant. Counts released to the supernatant would indicate methyltransferase activity, that is radioactively labelled methyl groups being transferred from DNA to protein. Varying amounts of AlkB protein were incubated with DNA substrates under different conditions. A summary is presented in Table 9. We did not detect methyltransferase activity of AlkB.

In vitro HPLC assays

The possibility that AlkB transfers the methyl group from one DNA base to another to generate an innocuous form of damage was considered. In order to investigate this model it was necessary to release the methylated bases from DNA and separate them by HPLC.

As no counts were released in the DNA glycosylase assays, presumably they all still resided in the DNA pellet. Methylated purines and pyrimidines remaining in the DNA were analysed by different procedures. Acid hydrolysis was used to release purines from the pellet, and enzymatic hydrolysis to release the

LABELLED DNA SUBSTRATE	ALKB PROTEIN	CRUDE EXTRACT (5 µg)	$MgCl_2$ (5 mM)	NaCl (50 mM)	RESULT
[^{14}C] MMS M13	0 - 0.2 µg (0 - 8 pmol)		- + - +	- - + +	Negative
[^{14}C] MMS poly (dA)	0 - 0.2 µg (0 - 8 pmol)		- + - +	- - + +	Negative
[^{3}H] DMS poly (dA)	0 - 0.1 µg (0 - 4 pmol)		- + -	- - +	Negative
[^{14}C] MMS poly (dC)	0 - 0.5 µg (0 - 20 pmol)		- + - +	- - + +	Negative
[^{3}H] DMS poly (dA)/ poly (dT)	0 - 1 µg (0 - 42 pmol)		- + -	- - +	Negative
[^{14}C] Poly (dC)/ poly (dI)	0 - 0.5 µg (0 - 20 pmol)		+	+	Negative
[^{14}C] MMS M13 [^{14}C] Poly (dA) [^{14}C]] Poly (dC)		AB1157/ BS87/ BS118 (AlkB ↑)	- +	- +	Negative
[^{14}C] MMS M13	0.12 µg (5 pmol)	BS87	- +	- +	Negative

TABLE 8: SUMMARY OF DNA GLYCOSYLASE ASSAYS

Various DNA substrates were incubated with purified AlkB protein or crude cell extracts at 37°C, 1 hr. The DNA was precipitated, and the supernatant assayed for soluble counts to indicate the presence of glycosylase activity.

LABELLED DNA SUBSTRATE	ALKB PROTEIN	$MgCl_2$ (5 mM)	NaCl (50 mM)	RESULT
[^{3}H] DMS poly (dA)	0 - 0.2 µg (0 - 8 pmol)	- + - +	- - + +	Negative
[^{14}C] MMS poly (dC)	0 - 0.1 µg (0 - 4 pmol)	- + - +	- - + +	Negative
[^{3}H] DMS poly (dA)/ poly (dT)	0 – 0.5 µg (0 - 20 pmol)	- + - +	- - + +	Negative
[^{14}C] poly (dC)/ poly (dI)	0 – 0.5 µg (0 - 20 pmol)	- + - +	- - + +	Negative

TABLE 9: SUMMARY OF METHYLTRANSFERASE ASSAYS

Various labelled DNA substrates were incubated with purified AlkB protein at 37°C, 30 min, followed by DNA and protein precipitation. The pellet was digested with proteinase K, 37°C, 2 hr to release all amino acids to the supernatant. The remaining DNA was re- precipitated and the supernatant assayed for soluble counts, to indicate the presence of methyltransferase activity.

pyrimidines. Samples were loaded onto a SCX column, and eluted using a mixed buffer of ammonium formate and methanol. Co- chromatography with unlabelled markers allowed identification of the labelled peaks. All methylated bases carry a different charge and have different retention times on the SCX column, (Figures 23B, 24B and 25B). We were able to separate all the known DNA methylated bases, and looked for appearance or disappearance of a peak in the presence of AlkB. HPLC analyses were carried out on most of the pellets obtained from the glycosylase assays, and a summary of all the HPLC runs are presented in Table 10. Thus, various DNA substrates were incubated with purified AlkB protein or cell extracts under different conditions. Cell extracts were tested in case certain cofactors were needed for AlkB activity which would not be present in the purified AlkB preparations. Examples of chromatograph outputs of HPLC runs are presented in Figures 23 - 25. Whether analysing the processing of modified adenines by purified AlkB protein, (Figure 23A), or modified adenines and guanines by cell extracts, (Figure 24A), or methylated deoxycytidine by purified AlkB, (Figure 25A), the peaks were always identical. These results indicated that AlkB did not process these methylated bases in the *in vitro* conditions analysed.

The HPLC, glycosylase and methyltransferase studies were done *in vitro*, and it is possible that the AlkB protein was not active in the conditions used. Thus experiments to analyse *in vivo* processing of modified bases were set up. M13 SS DNA bacteriophage was treated with [^{3}H] DMS and used to infect wild type (BS141) and *alkB* mutant (BS143) F' strains. DNA was rescued from these cells and analysed by HPLC. To date, methylated purines in the bacteriophage DNA retrieved from wild type and *alkB* mutant cells did not show any detectable difference, (data not shown).

To summarise, presented in this chapter are *in vitro* analyses that seem to rule out any DNA glycosylase, DNA methyltransferase or other type of activity that

could modify a lesion for AlkB, in the experimental conditions tested.

LABELLED DNA SUBSTRATE	ALKB PROTEIN	CRUDE EXTRACT (5 μg)	$MgCl_2$ (5 mM)	NaCl (50 mM)	RESULT
[^{14}C] MMS M13	0 - 0.12 μg (0 - 5 pmol)		-	-	Negative
[^{14}C] MMS poly (dA)	0 - 0.12 μg (0 - 5 pmol)		-	-	Negative
[^{3}H] DMS poly (dA)	0 - 0.1 μg (0 - 4 pmol)		- + -	- - +	Negative
[^{14}C] MMS poly (dC)	0 - 0.5 μg (0 - 20 pmol)		-	-	Negative
[^{3}H] DMS poly (dA)/ poly (dT)	0 - 1 μg (0 - 42 pmol)		+ -	- +	Negative
[^{14}C] MMS poly (dC)/ poly (dI)	0 - 0.5 μg (0 - 20 pmol)		-	-	Negative
[^{14}C] MMS M13 [^{14}C] MMS poly (dA) [^{14}C] MMS poly (dC)		AB1157/ BS87	-	-	Negative

TABLE 10: SUMMARY OF HPLC ASSAYS

Various labelled DNA substrates were incubated with purified AlkB protein or crude cell extracts at 37°C, 30 min, followed by DNA precipitation. The pellets were hydrolysed and the samples then examined via HPLC.

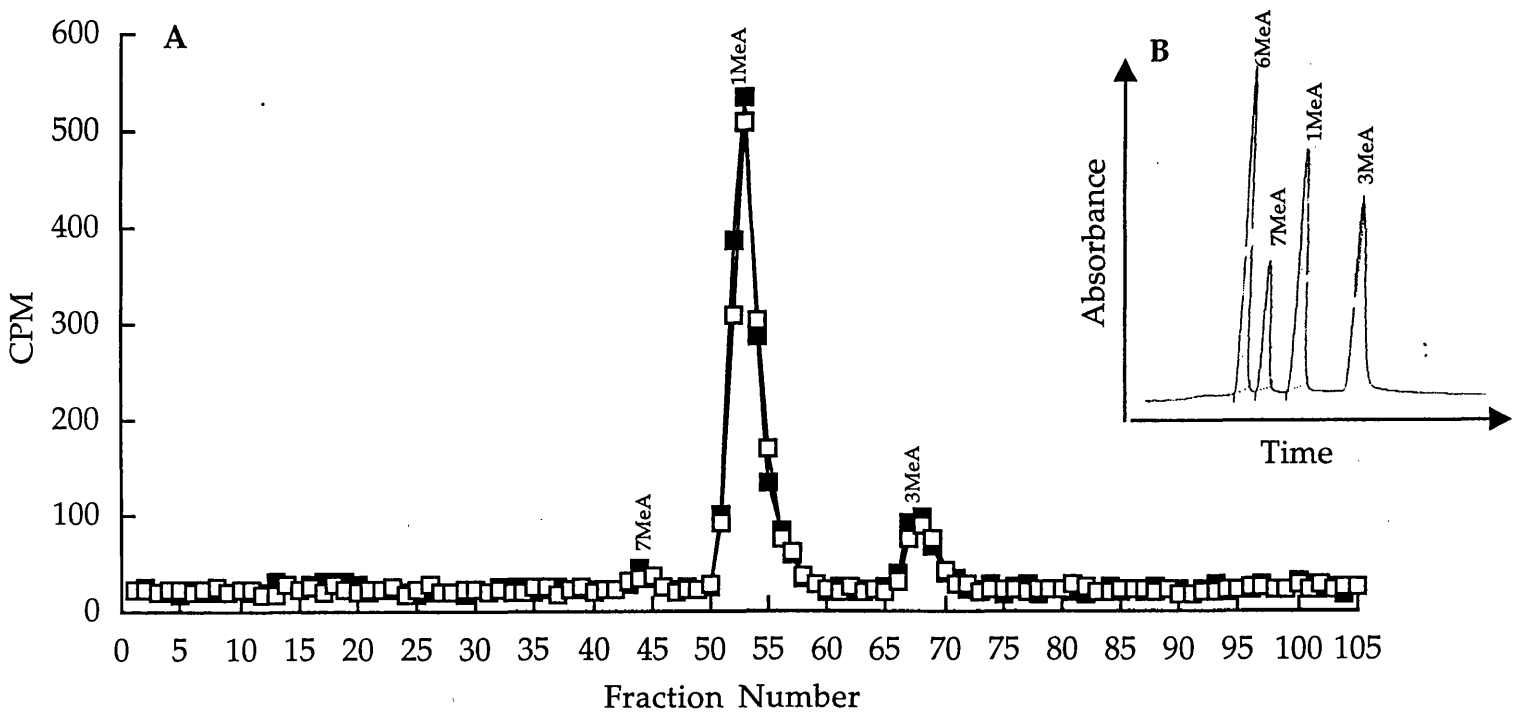


FIGURE 23: ALKB AND [^{14}C] MMS TREATED POLY (dA)

(A) *AlkB has no detectable effect on MMS treated poly (dA)* - [^{14}C] MMS treated poly (dA) (15mM) was incubated with 0.12 μg (5 pmol) AlkB, and then acid hydrolysed (0.1M HCl) to release the free bases. Samples were analysed by HPLC on a SCX column using a mixed buffer system of ammonium formate and methanol. Fractions were collected every 31 seconds, and the peaks quantitated by scintillation counting. : ■: + AlkB; □ : - AlkB.

(B) *HPLC standard markers* - Standard markers were run through the HPLC system, and the retention times noted for each to allow identification of the peaks obtained in (A).

Retention times (min): 6MeA (12.65); 7MeA (14.53); 1MeA (17.29); 3MeA (22.33).

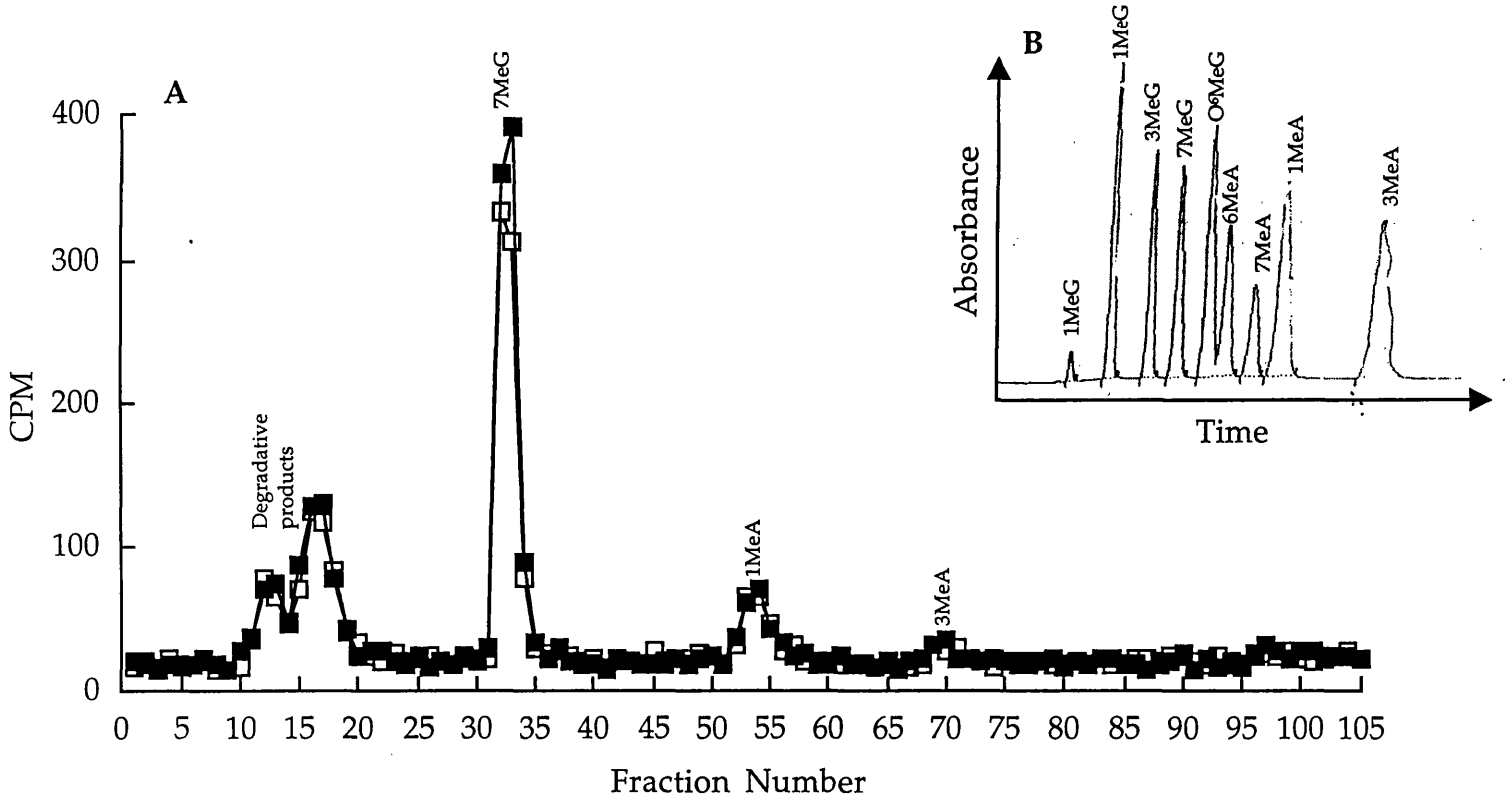


FIGURE 24: ALKB AND [^{14}C] MMS TREATED M13 SS DNA

(A) *AlkB crude extracts have no detectable effect on MMS treated M13 SS DNA* - [^{14}C] MMS treated M13 SS DNA (15mM) was incubated with 5 µg crude extracts, and then acid hydrolysed (0.1M HCl) to release the free bases. Samples were analysed by HPLC on a SCX column using a mixed buffer system of ammonium formate and methanol. Fractions were collected every 31 seconds, and the peaks quantitated by scintillation counting. ■: AB1157 (wild type); □: BS87 (alkB).

(B) *HPLC standard markers* - Standard markers were run through the HPLC system, and the retention times noted for each to allow identification of the peaks obtained in (A).

Retention times (min):
1MeG (6.20 + 8.44); 7MeG (10.81); 3MeG (12.52); O^{6}MeG (14.38); 6MeA (15.52); 7MeA (17.20); 1MeA (18.85); 3MeA (24.53).

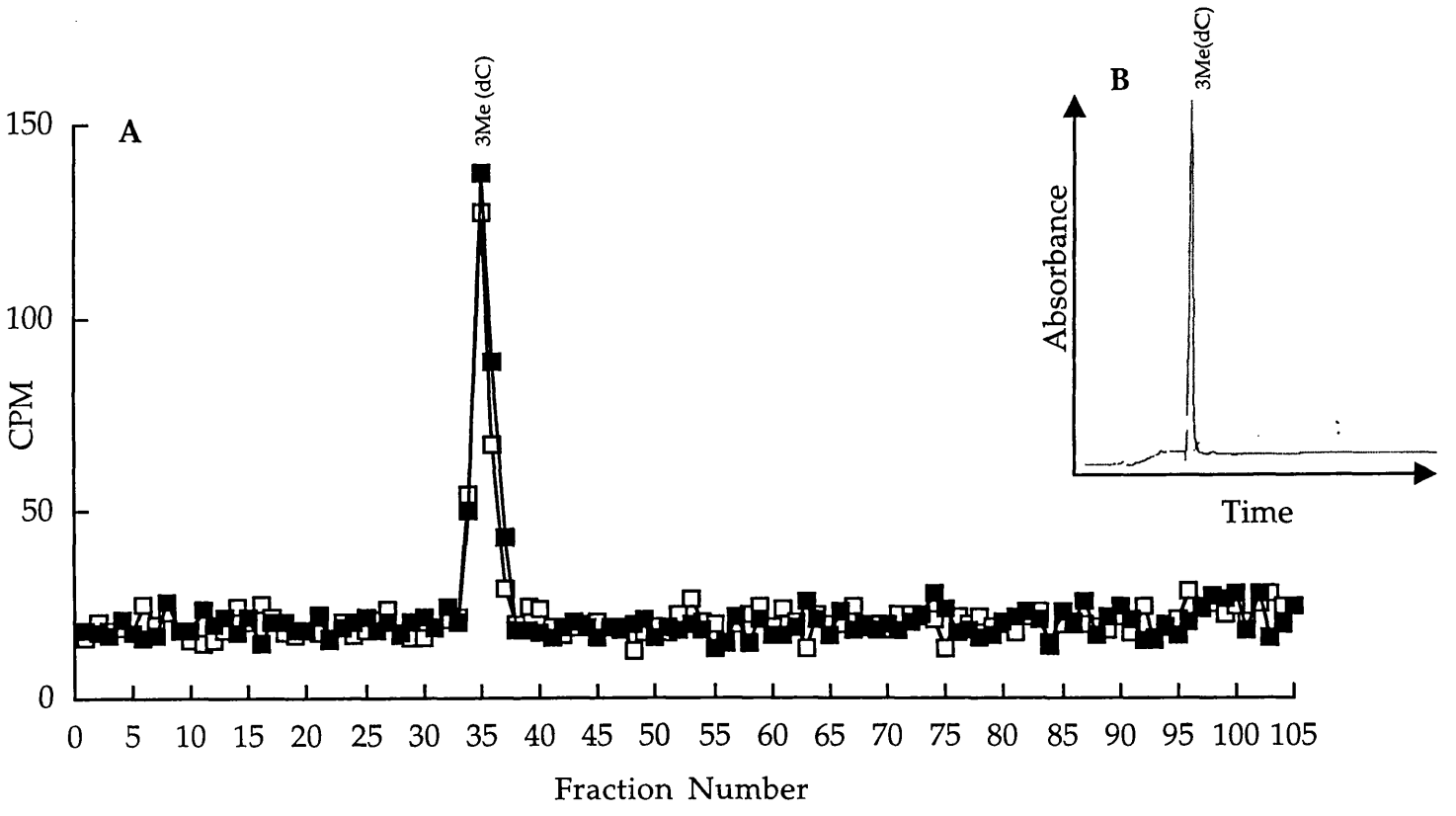


FIGURE 25: ALKB AND [^{14}C] MMS TREATED POLY (dC)

(A) *AlkB has no detectable effect on MMS treated poly (dC)* - [^{14}C] MMS treated poly (dC) (15mM) was incubated with 0.5 µg (20 pmol) AlkB in the presence of NaCl (50mM) and $MgCl_2$ (5mM). Samples were enzymatically hydrolysed (DNaseI, snake venom phosphodiesterase & alkaline phosphatase) to release deoxynucleosides. The samples were analysed by HPLC on a SCX column using a mixed buffer system of ammonium formate and methanol. Fractions were collected every 31 seconds, and the peaks quantitated by scintillation counting. ■: + AlkB; □ : - AlkB.

(B) *HPLC standard marker* - Standard marker 3′ methyl 2′deoxycytidine methosulfate was run through the HPLC system, and the retention time noted to allow identification of the peak obtained in (A).

Retention time (min): 3Me(dC) (10.54).

Chapter 8

CHAPTER 8

Discussion & Conclusion

The role of AlkB has eluded the investigators for over fifteen years. In this chapter all the results are discussed, and the implications of each towards identifying functional role(s) for AlkB considered. The AlkB protein is a 24kDa protein, expressed as part of the adaptive response of *E. coli* towards alkylation damage. *E. coli alkB* mutant cells are sensitive to the cytotoxic affect of S_N2 alkylating agents such as MMS. Conservation through evolution from bacteria to man indicates that AlkB must hold an important role(s).

AlkB does not repair the toxic lesion 3MeA or DNA strand breaks in vivo

Initial investigations in chapter 3 examined and ruled out a role for AlkB in repair of a major toxic lesion 3MeA or in repair of DNA strand breaks *in vivo*. DNA can exist in three forms, the A, B or Z form. In the cell DNA is generally in the B form with two grooves called the major and minor grooves. These grooves allow DNA interactions with other molecules (Mathews and van Holde, 1990). Ligands that bind DNA can be divided into two categories including those that are 'intercalators' and can insert their aromatic ring between two adjacent base pairs, or those that are 'groove binders' and can bind either DNA groove (Helene, 1998). MMS methylates DNA at many sites in both the major and minor groove of DNA. To limit the number of lesions formed a compound was designed called $MeOSO_2(CH_2)_2$-Lex (Me-Lex), to target the minor groove of DNA. This compound is composed of subunits of N'methylpyrrolecarboxamide, referred to as lexitropsins (Lex) attached to a methyl sulfonate ester, and binds to specifically target A/T rich regions of DNA predominantly producing the toxic lesion 3MeA (Zhang *et al.*, 1993). Lesion 3MeG that is also generated by MMS in the minor groove of DNA and is

known to be a lethal lesion was not produced by Me-Lex in any significant amount to result in any of the observed cytotoxicity. The ratio of 3MeG to 3MeA is 1:30 in MMS compared to 1:400 in Me-Lex due to the A/T preferential binding of Me-Lex [Personal communication, B. Gold]. The relative abundance of minor to major groove adduction was approximately 600 fold increased with Me-Lex compared to MMS (Kelly *et al.*, 1999). As lesions generated by the two compounds differed with Me-Lex producing a narrower spectrum of methylated bases, the role of AlkB to process the lesion 3MeA in the minor groove of DNA was investigated. $MeOSO_2(CH_2)_2$-Lex sensitivity of various *E. coli* strains was compared to that of MMS. Single mutants *alkB* and *alkA1* were not sensitive to Me-Lex. The *tagA1* gene product, 3-methyladenine-DNA glycosylase I that is constitutively produced in *E. coli* cells was presumably sufficient to deal with the 3MeA generated by Me-Lex. This assumption proved to be correct as a *tagA1 alkA1* double mutant was sensitive to Me-Lex. The triple mutant *tagA1 alkA1 alkB* was not any more sensitive to Me-Lex than the double mutant. Thus we conclusively showed that AlkB did not repair toxic lesion 3MeA produced in the minor groove of DNA.

For comparison, treatment of *E. coli* strains with MMS showed single mutants *alkA1* and *alkB* to be sensitive. The double mutant *tagA1 alkA1* showed increased sensitivity, and the triple mutant more so. These observations proved that AlkB did not have a direct role in repair of 3MeA, but processed a different lesion generated by MMS but not Me-Lex in either the major or the minor groove of DNA.

Damage resulting in scission of the DNA backbone results in strand breaks. Monofunctional alkylating agents primarily do not cause strand breaks but the base modification by alkylation damage can weaken the N-glycosylic bond. This can lead to base depurination/ depyrimidination, and subsequent cleavage of the AP site to eventually cause a DNA break (Horvathova *et al.*, 1998). DNA

treated with DMS was reported to also undergo spontaneous and non- random DNA cleavage. The chemical basis of this cleavage was unknown, thus DNA strand breaks may occur by a mechanism other than cleavage of abasic sites (Dolle and Stratling, 1989). Using MMS to treat *E. coli* wild type (HK80) and *alkB* mutant (HK82) cells carrying plasmid pET15b, we were unable to detect the formation of DNA strand breaks *in vivo*. Plasmids generally exist in a closed circular form. If strand breaks had occurred on treating the strains with increasing MMS doses, there would have been an increased shift of the closed circular to open circular form of the plasmid. At the same time survival of the *alkB* mutant cells was observed to decrease dramatically. Approximately 30 copies of pET15b (5.71 Kb) exist per cell, and created a plasmid target of 1.7 x 10^5 base pairs. This target was smaller than the chromosome target of 4.6 x 10^6 base pairs. DNA strand breaks induced by MMS in chromosomal DNA were detected previously by alkaline sucrose gradient sedimentation at a level that would have been detected in our experiments (Green *et al*, 1974). The breaks detected by Green *et al* were possibly a result of cleavage of AP sites by alkali. Thus using gentler DNA extraction methods suggested that if DNA strand breakages were caused by the MMS treatment *in vivo* they were rare events and were probably not the primary lesion repaired by the AlkB protein.

Abasic sites in DNA are potentially toxic lesions, and secondary lesions are associated with these sites. If the base opposite an abasic site is a pyrimidine it can cause the DNA helix to collapse, and if it is a purine, interstrand stacking interactions can occur (Friedberg *et al.*, 1995). The aldehyde group of an apurinic site can react with an amino group on the opposite strand to form a Schiff base, a condensation product between an amino and carbonyl group (Goffin and Verly, 1983). Thus secondary lesions associated with AP sites whether interstrand or intrastrand DNA- DNA crosslinks or DNA- protein crosslinks can inhibit DNA, RNA, and protein synthesis in dividing cells. As part of the initial investigations in our laboratory, we concluded that AlkB did

not process abasic toxic lesions or secondary lesions that could possibly arise at those sites (Dinglay *et al.*, 1998).

AlkB is involved in processing damaged SS DNA

All past *in vitro* AlkB studies tested for a repair function using DS DNA damaged by MMS. Strikingly seen is that MMS generates different lesions to MNU in SS DNA, (Table 1). Base lesions 1MeA and 3MeC are produced in high amounts in SS DNA, and no repair protein has as yet been associated with correction of these two lesions. Experiments in chapter 4 revealed a dramatic defect in *alkB* mutants to process damaged SS DNA. This observation was a major step forward towards identification of possible role(s) for AlkB. Defective processing of damaged SS DNA by AlkB was specific to damage generated by the S_N2 alkylating agents, that is MMS, MeI and DMS. MeI is classified as an S_N2 agent, able to methylate both DNA and protein (Oh *et al.*, 1996; Vaughan *et al.*, 1993). Earlier investigations reported a functional independence for AlkB (Chen *et al.*, 1994). Our experiments support this observation. We ruled out AlkB interaction with other proteins involved in removal of alkylation damage, namely the Ada, AlkA and Tag proteins. Interaction with other known DNA repair pathways including NER, MMR and SOS responses was ruled out too.

The *uvrA* and *mutS* gene products act at first stage recognition of DNA damage in NER and MMR (Sancar, 1996; Jiricny, 1998). It was considered that AlkB could be involved in replication past a blocking lesion with subsequent removal of the lesion by NER. Another possibility was that a mispaired base was generated in the replication fork in the absence of *alkB*, and was then recognised by the MMR pathway. Repeated attempts to remove it by MMR could result in cell death, a phenotype that is seen on treatment of *alkB* mutant cells with MMS. The process would be analogous to the mechanism of methylation tolerance in eukaryotic cells as was described in section 1.3 of the

introduction. *E. coli uvrA* and *mutS* mutants were not defective in processing MMS damaged SS DNA, and the double mutants *uvrA alkB* and *mutS alkB* were no more defective than the single *alkB* mutant ruling out interaction with both these DNA repair pathways.

The *umuC* and *umuD* genes form an operon, and are induced as part of the SOS response to DNA damage. RecA protein may attract the Umu proteins to the damaged site on SS DNA at the replication fork, which then allows the UmuD'$_2$C polymerase activity to replicate past the lesion in an error prone manner by nucleotide misincorporation opposite the lesion (Smith and Walker, 1998). AlkB did not stimulate umuDC in an error prone bypass function, as defective host cell reactivation of MMS treated SS DNA bacteriophage was not seen in the *umuDC* mutants. Translesional bypass involves an increased mutation frequency, and as will be discussed later, AlkB activity did not result in increased MMS induced mutagenesis. Thus, direct interaction of AlkB with the SOS response was ruled out.

Interaction of AlkB with RecA was also investigated. RecA modulates many DNA repair pathways, and is involved in the SOS response as well as recombinational repair. We observed that a *recA* mutant was slightly defective in reactivation of damaged SS DNA bacteriophage compared to the wild type strain. The *recA alkB* double mutant was also more defective than the *alkB* single mutant. These differences indicated an additive affect, suggesting that RecA and AlkB worked independently. This directly ruled out all involvement of AlkB in recombinational repair as RecA is the principal protein involved in mediating the process. RecA may provide a functional backup for AlkB if the amount of DNA damage became excessive, and therefore have a minor role in processing damaged SS DNA.

Since AlkB processed damaged SS DNA, we hypothesised that *alkB* mutant

cells might be more sensitive to DNA damage during rapid growth, as more single stranded regions would be present at replication forks and regions of transcription. This proved true as *alkB* cells in exponential phase of growth were more sensitive to MMS compared to cells in stationary phase. Stationary phase *alkB* cells showed an intermediate survival between *alkB* in exponential phase of growth and wild type cells. Although incompletely understood, cryptic DNA turnover occurs quite extensively in stationary phase cells and may explain our intermediate result (Bridges, 1996; Bridges, 1997). Thus, AlkB processes damaged SS DNA, and may work most actively during replication.

AlkB mutants show a low mutation frequency

The *E. coli* spontaneous mutation rate is 2 x 10^{-10} per base pair during replication (Drake, 1969). Increased mutation rates are prevented by repair of replication errors and damage to DNA. AlkB action did not enhance MMS induced mutagenesis (Chapter 6). *E. coli* strains CC101 - CC106 carry different point mutations in the F' *lacZ* gene. By transduction, *alkB* mutant derivatives of each strain were created, and designated CC101 *alkB* - CC106 *alkB*. CC102 *alkB*, CC104 *alkB* and CC105 *alkB* strains treated with MMS showed a low revertant frequency to the $LacZ^+$ phenotype compared to their respective parent strains. The mutations to $LacZ^+$ were probably generated by an error prone bypass of polymerase III blocking lesions. All MMS induced mutation frequencies in *alkB* were low in comparison to a CC102 *Δ(ada alkB) ogt* mutant (Miller, 1998). The *ada* and *ogt* gene products encode DNA methyltransferase activities that correct O^6MeG DNA lesions. A lack of repair of O^6MeG results in a high frequency of G:C to A:T transition mutations (Mackay *et al.*, 1994). The low MMS induced mutations in an *alkB* mutant confirmed that its action was not involved in error prone translesional bypass, but in an efficient and error free process.

Lack of the *alkB* gene product in CC102 *alkB*, CC104 *alkB*, and CC105 *alkB* all

resulted in a transition or transversion to an adenine base. Preferential incorporation of adenines opposite a lesion is known as the 'A-rule'. In *E. coli* adenine is the primary DNA base inserted, as it can stack in an intrahelical configuration so as not to disturb the DNA helix. If adenine bases are not available, then guanines followed by cytosine or thymine bases are used (Randall *et al.*, 1987; Strauss, 1991). Insertion of a DNA base would help bypass the lesion allowing DNA replication to continue. Thus if AlkB were absent then error prone replication switches on, resulting in adenine incorporation to bypass a lesion probably by the error prone polymerase.

We had hoped to pinpoint the damaged base bypassed using CC101 - CC106 strains, but no particular point mutation occurred at a notably higher frequency than the others.

AlkB binds SS DNA and its binding is enhanced by DNA methylation

The interaction of AlkB and DNA was examined. We conclusively showed that AlkB bound to DNA by three different binding techniques. Binding to DNA cellulose initially showed that AlkB did recognise and bind to DNA. Unable to distinguish an affinity for SS DNA over DS DNA or vice versa, more quantitative techniques were carried out.

Gel shift assays detect DNA bound to protein by a mobility shift during migration through a polyacrylamide gel (Taylor *et al.*, 1994), and also indicated that AlkB did bind to DNA. Scanning of radioactively labelled DNA bands in the gel revealed that AlkB showed a greater affinity towards SS DNA than DS DNA. Filter binding assays were used to quantitate the binding more accurately and study the binding properties of AlkB with various DNA substrates damaged with MMS. This procedure was first used to examine RNA- protein interactions in 1964, and then in 1966 by Jones and Berg to study protein interactions with DNA (Nirenberg and Leder, 1964; Jones and Berg, 1966). The technique is

rapid and the results reproducible, the only disadvantage being that it does not pick up the stoichiometry of AlkB binding to DNA, unlike the gel shift assays (Fried and Crothers, 1981; Stockley, 1994). The results showed that AlkB bound both SS and DS DNA, with higher affinity for SS DNA. AlkB also bound methylated better than unmethylated DNA, with a higher affinity for methylated SS DNA. AlkB showed a higher affinity for the unmethylated pyrimidine substrates poly (dC) and especially poly (dT). This feature of binding has been previously reported for other proteins, and attributed to secondary structural features of the oligonucleotides rather than preferential binding to a specific DNA sequence (Weiner *et al.*, 1975). Homopolymers of adenine bases known to form strong stacking structures make the oligonucleotide less flexible, and so a faster dissociation rate of poly (dA) from the protein would explain the lower binding affinity observed (Dewey and Turner, 1980). Whichever substrate was used there was a two- fold increase in affinity for binding to methylated SS DNA compared to its unmethylated form. Thus a particular substrate for AlkB was not pinpointed, as there was no greatly enhanced binding towards methylated poly (dA), poly (dC) or poly (dT).

The DS DNA oligonucleotides showed a lower affinity of binding to AlkB compared to SS DNA oligonucleotides. Methylated poly (dA)/ poly (dT) and poly (dI)/ poly (dC) showed greater binding compared to the general DS DNA substrate. Methylated poly (dI)/ poly (dC) binding was higher than the general two- fold increase. One could argue that in this case only, a slipped structure may have formed resulting in a bubble structure. Some proteins have affinity towards such structures such as high mobility group (HMG) proteins (Grosschedl *et al.*, 1994; David-Cordonnier *et al.*, 1998). Thus AlkB may recognise DS DNA with regions of single strands such as at replication forks. If there were damage in such a structure, would this further enhance AlkB binding affinity? Further investigations are required to test this possibility.

Many nucleases require and act proficiently in the presence of magnesium ions (Kessler and Manta, 1990). Had AlkB been a nuclease, we would have expected complete degradation of the oligonucleotide substrate in the presence of magnesium, unless a specific recognition sequence was required. The His-tagged preparation of AlkB showed a very weak nuclease activity, but this has been noted before for other purified His- tagged proteins [Personal communication – Dr. Steve West]. Thus, trace of nuclease activity was ascribed to a contaminating *E. coli* activity, and the AlkB protein itself probably was not a nuclease.

Nucleotides and nucleosides are able to bind many proteins non- specifically (Kalf, 1981). The gel shift assays showed that AlkB binding to DNA substrates was specific, as no binding of DNA to BSA and a high affinity of SS DNA towards SSB protein were observed. DNA- protein complexes can interact by sequence recognition, by strand conformations, or by DNA groove geometries (Berg and von Hippel, 1988). SS DNA binding proteins such as SSB are involved in replication. They increase the accuracy of DNA synthesis, protect DNA from nuclease attack, and act as a helix destabilising proteins (Lohman *et al.*, 1988). We can suggest that AlkB is a DNA damage recognition protein, particularly of damage in SS DNA. With information gained so far, it was reasoned that AlkB may recognise, bind and process lesions in DNA replication forks. As the DNA binding affinity of AlkB was weak compared to SSB, recognition maybe momentary allowing enough time for activating the repair processing machinery.

AlkB does not possess detectable DNA glycosylase or methyltransferase activities, or ability to transform a lesion to an innocuous form of damage

Enzymatic analyses carried out *in vitro* (Chapter 7) indicated that AlkB did not possess any DNA glycosylase or methyltransferase activities, or an ability to transform a lesion into a different innocuous form. By incubating purified AlkB

protein or cell extracts with various DNA substrates, we examined the possible involvement of AlkB in processing lesions 1MeA and 3MeC. The DNA glycosylases AlkA and Tag remove the lesion 3MeA from DNA, with AlkA possessing a broader substrate specificity. Both the enzymes can remove damage from SS DNA. AlkA compared to Tag is able to do this more efficiently, but neither of the two enzymes remove the lesion 1MeA (Bjelland and Seeberg, 1996). We showed that AlkB was also not a DNA glycosylase repairing lesions in SS DNA such as 1MeA or 3MeC. Furthermore AlkB did not have DNA methyltransferase activity. 3MeC is a potent inhibitor of DNA synthesis *in vitro*, preventing chain elongation by blocking DNA polymerase I, and is also promutagenic during *in vitro* RNA synthesis (Saffhill, 1984). *AlkB* mutants show a low mutation frequency when exposed to MMS, so could AlkB recognise 3MeC or 1MeA during DNA replication and convert it into a non-cytotoxic base? For example could 1MeA be converted to 6MeA in what is known as the Dimroth rearrangement, or could 3MeC be deaminated to 3MeU? HPLC studies were carried out to investigate this possibility but indicated that transferral of these lesions into an innocuous form of damage did not occur in the conditions tested.

It is interesting to note a recent report of adducts 1-(2-Hydroxypropyl)adenine (1-HP-adenine) and 3-(2-Hydroxypropyl)cytosine (3-HP-cytosine) that formed in rats on propylene oxide (PO) treatment, a monofunctional alkylating agent known to be genotoxic and carcinogenic. These were bulkier forms of our candidate lesions 1MeA and 3MeC. Upon incubation *in vitro* of PO treated DNA with protein extracts from HeLa cells, the authors observed an unknown enzymatic removal of the lesion 3-HP-cytosine (Plna *et al.*, 1999). Enzymatic repair of similar bulky lesions, cyclic 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC) has been previously reported. Both of these lesions were found to be repaired by a DNA glycosylase activity that recognised the damage in DS DNA. In *E. coli* AlkA repairs the lesion εA, with an analogous activity

AAG occurring in humans. Repair of lesion εC was attributed to the bacterial double- stranded uracil- DNA glycosylase (ds UDG) in *E. coli*, and the homologous mismatch- specific thymine- DNA glycosylase in humans, (hTDG) (Saparbaev and Laval, 1998). Therefore, removal of the reported 3-HP-cytosine lesion was possibly due to hTDG activity. Our studies were limited to our experimental conditions tested, but we suspect that AlkB has a different activity to the above observations, as no DNA glycosylase activity was found in our *in vitro* studies.

AlkB's exact functional role is still an unknown area. I have presented work that has made a positive advance towards solving this problem. Considering all the observations, I can hypothesise a model for AlkB function.

This work suggests that AlkB is a protein that specifically can bind and process damaged SS DNA. Its function is independent of most of the known DNA repair pathways. A suggested function for AlkB could be a damage inducible polymerase activity comparable to related proteins DinB or the UmuD'$_2$C complex of the SOS response. DinB encoded polymerase activity (Pol IV) is devoid of proofreading activity, is independent of RecA and UmuDC, and has the ability to extend past bulged (misaligned) DNA structures. Its activity is error prone, forming a slipped intermediate structure which results in -1 frameshift mutations giving rise to replication products one nucleotide shorter (Friedberg and Gerlach, 1999; Wagner *et al.*, 1999). It has recently been shown that UmuC also contains an active site for polymerase activity. UmuD'$_2$ the stimulatory subunit binds to form UmuD'$_2$C and gives rise to polymerase activity (Pol V). This polymerase bypasses by base misincorporation leading to increased mutation frequencies (Tang *et al.*, 1999; Woodgate, 1999). We did not observe an increased mutation frequency associated with AlkB function. Thus the action of AlkB could encode an enzyme such as that of a polymerase or one with excision activity, but would have to be error free with non-

promutagenic affects. Absence of AlkB could possibly stimulate $UmuD'_2C$, leading to error prone replication. This would tie in with our low mutation frequencies observed in the absence of AlkB. *In vitro* techniques for detection of translesional synthesis using single site- specific lesions in SS DNA could be used to investigate error free bypass by AlkB of our candidate lesions 1MeA and 3MeC (Wang *et al.*, 1997). However, no sequence homology between AlkB and any of the recently investigated DNA polymerases for lesion bypass has been detected. Also, preliminary assays conducted in our laboratory did not detect any polymerase activity for AlkB, although this is still under investigation.

AlkB protein is expected to have an error free DNA processing activity. Its activity may occur on SS DNA at blocked replication forks, where it could prevent cell death by efficient DNA processing. AlkB expression has been linked with the cell cycle in *Caulobacter crescentus*, its expression increasing prior to DNA replication (Colombi and Gomes, 1997). AlkB however could still have a DNA repair function that we have failed to detect, and it has been recently shown by sequence comparisons to have an apparent hydrolase domain (Aravind *et al.*, 1999). A diagrammatic model for possible AlkB roles is presented in Figure 26. The present work could serve as a starting point for further investigations towards determining AlkB function, and experiments continue in our laboratory. Immediate studies include understanding how AlkB recognises damage, and whether it does so in the SS DNA form or in DS DNA. We are also trying to narrow down the lesion or lesions that AlkB could process, and from this develop an *in vitro* assay for its function.

?A BLACK BOX?

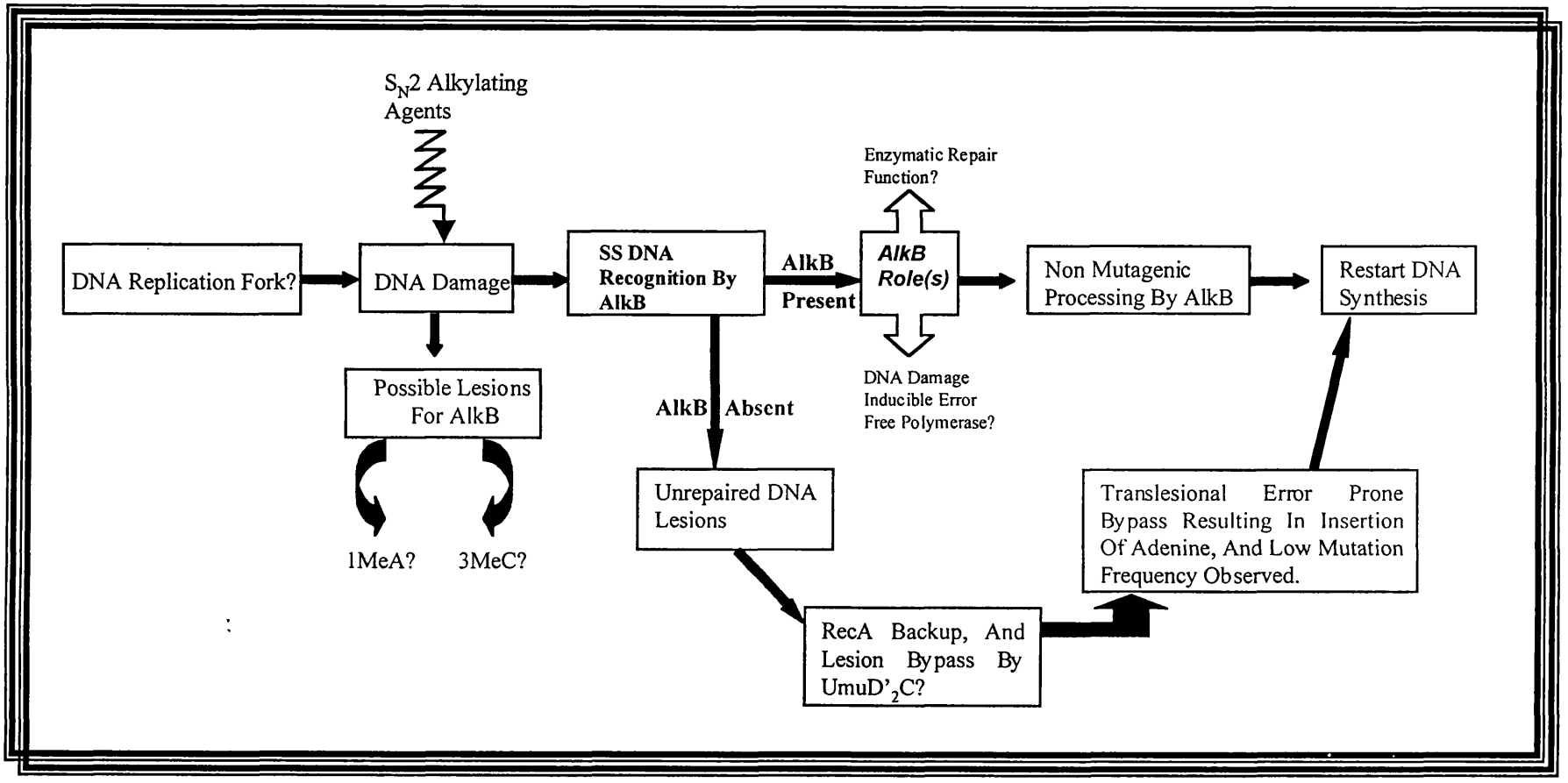


FIGURE 26: DIAGRAMMATIC MODEL OF ALKB IN PROCESSING DNA DAMAGE

CONCLUSION

1. AlkB processes damaged SS DNA.
2. AlkB is sensitive to damage by S_N2 alkylating agents.
3. AlkB binds DNA especially SS DNA and methylated SS DNA.
4. AlkB processes damage in an error free manner.
5. AlkB is independent of known DNA repair pathways tested.

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Repair in *Escherichia coli alkB* mutants of abasic sites and 3-methyladenine residues in DNA

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Abstract

Escherichia coli alkB mutants are sensitive to methyl methanesulfonate and dimethylsulphate, and are defective in the processing of methylated DNA. The function of the AlkB protein has not been determined. Here, we show that *alkB* mutants are not defective in repairing several different types of potentially toxic DNA lesions that are known to be generated by MMS, including apyrimidinic and apurinic sites, and secondary lesions that could arise at these sites (DNA–protein cross-links and DNA interstrand cross-links). Also, *alkB* mutants were not sensitive to $MeOSO_2$-$(CH_2)_2$-Lex, a compound that alkylates the minor groove of DNA generating primarily 3-methyladenine. © 1998 Elsevier Science B.V.

Keywords: *Escherichia coli alkB* mutant; DNA methylation

1. Introduction

DNA alkylating agents occur endogenously, environmentally and are also used as chemotherapeutic agents [1–3]. They generate many forms of DNA damage, and as a result are toxic, mutagenic and carcinogenic. N^7-methylguanine is the most abundant, although relatively innocuous form of damage generated by most DNA methylating agents, including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS). The major toxic lesion that blocks DNA replication is 3-methyladenine (3-meA), and the major mispairing, and therefore mutagenic lesion is O^6-methylguanine (O^6-meG) [4]. Cells are protected against alkylation damage by DNA repair enzymes, some of which are specific for the removal of alkylated bases from DNA. Ubiquitous 3-meA-DNA glycosylases excise 3-methyladenine from DNA, and the resulting non-coding abasic sites are rapidly incised by apurinic endonucleases, filled by DNA polymerases and ligated in the base excision repair pathway [5]. O^6-meG-DNA methyltransferases are also ubiquitous and directly demethylate O^6-meG by transferring the methyl group onto one of their own cysteine residues. Guanine is thereby directly regenerated in DNA, and no further activities are required to complete the repair process [6,7].

Escherichia coli has two 3-meA-DNA glycosylases (Tag and AlkA), and two O^6-meG-DNA methyltransferases (Ogt and Ada). The AlkA glycosylase

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has a wider substrate range than the Tag enzyme, and can excise not only 3-methyladenine but also 3-methylguanine, O^2-methylpyrimidines and 7-methylguanine [8–10]. The *tag* and *ogt* genes are expressed constitutively whereas *alkA* and *ada* are induced on exposure of cells to DNA methylating agents. This adaptive response is regulated by the Ada protein that functions in both DNA repair and as a transcriptional activator. Two additional genes, *alkB* and *aidB*, are induced as part of this response [6]. The AidB protein has amino acid identity to several mammalian acyl coenzyme A dehydrogenases but its precise role is unclear [11]. AlkB mutants are sensitive to the toxic effects of MMS and dimethylsulphate (DMS), and are slightly sensitive to MNNG [12,13]. A role for the AlkB protein in processing of methylated DNA is suggested by the decreased survival of MMS-treated bacteriophage in an *alkB* mutant, but its specific function is undetermined [12]. Expression of the *alkB* gene in human cells conveys resistance to MMS toxicity. The *E. coli* AlkB protein is unlikely to be involved in complex formation with human proteins and probably acts independently [13]. Therefore, the AlkB protein probably does not target other DNA repair proteins to a site of damage, regulate gene expression or participate in replication of damaged DNA. The additive sensitivity to MMS caused by *alkB* and *alkA* mutations indicates that the gene products act on different lesions or in different pathways [14]. To date, no DNA glycosylase, DNA methyltransferase or DNA-dependent ATPase activities have been detected in purified AlkB protein preparations [15]. Also, comparison of activities in extracts of wild type and *alkB* mutant cells revealed no difference in formamidopyrimidine–DNA glycosylase activities, that excise imidazole ring opened N^7-methylguanine from methylated DNA, or endonuclease IV (apurinic endonuclease) activities [16]. AlkB has no significant amino acid sequence similarity to other known DNA repair proteins, although a short region of homology to oxidoreductases was suggested [15]. A human homologue of AlkB has been described [17], also homologues in the gram-negative bacteria *Caulobacter crescentus* [18] and *Salmonella typhimurium* [19].

MMS induces many different types of DNA lesions that can block DNA replication and result in cell death. These lesions may include methylated bases, DNA strand breaks, and secondary lesions such as abasic sites, DNA–protein cross-links and DNA interstrand cross-links. Abasic sites arise on spontaneous depurination or enzymatic excision by DNA glycosylases of methylated DNA bases. DNA–protein cross-links may occur as secondary lesions at abasic sites due to reaction of the aldehyde group on the 1′ position of the deoxyribose moiety with amino groups of proteins to form Schiff bases [20]. DNA interstrand cross-links may arise by the same type of mechanism in which abasic sites in one DNA strand react with amino-groups of bases in the opposite strand [21]. DNA chain breaks occur spontaneously in DMS-treated DNA, but the mechanism of their formation is unknown [22]. The aim of this work was to examine whether *alkB* mutants were defective in processing of one of these potentially toxic DNA lesions.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli K12 strains and plasmids are listed in Table 1. Bacterial transformants were isolated as described [29] and selected on L agar plates containing 50 μg/ml carbenicillin. SD1 (as GC4803 but *alkB117*::Tn3) was constructed by transduction of GC4803 with a thermoinduced P1 cml clr 100 lysate of BS87 (*alkB117*::Tn3). The transduction method was as described previously [30]. Transductants were selected and purified on 50 μg/ml carbenicillin at 42°C. The enhanced MMS sensitivity of the transductants compared with the parent strains was demonstrated by streaking 10 μl of cultures (A_{450} 0.4) across a gradient of 0 to 0.005% in a 10-cm square L agar plate and incubating at 37°C.

2.2. Preparation of λ_{gt} lysates

Strain AB1157, grown overnight at 37°C in LB broth, was centrifuged, resuspended and concentrated twofold in 10 mM $MgSO_4$. Approximately 1×10^5 to 1×10^6 λ_{gt} pfu were added to 0.5 ml cells and kept at 37°C for 30 min without shaking. LB broth (20 ml), 10 mM $MgSO_4$ was added, and the culture shaken at 37°C for 6 h. After addition of

Table 1
E. coli K12 strains and plasmids

Strains and plasmids	Genotype	Source or reference
Strains		
AB1157	*argE3 hisG4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-1 thi-1 rpsL31 supE44 tsx-33*	Laboratory stock
BS87	as AB1157 but *alkB117*::Tn3	[23]
HK80	as AB115 7 but *nalA*	[12]
HK82	as AB115 7 but *nalA alkB22*	[12]
BW9109	as AB1157 but *Δxth*	[24]
RPC501	as AB1157 but *nfo-1*::Kan^r *Δxth*	[24]
BD10	*thyA36 deoC ung-1*	[25]
CJ236	*dut-1 ung-1 thi-1 relA1*/pCJ105	[25]
MS23	as AB1157 but *alkA1 his*$^+$	[26]
GC4803	as AB1157 but X::Tn5 *tagA1 alkA1*	[27]
SD1	as GC4803 but *alkB117*::Tn3	This paper
Plasmids		
pAT153	Vector	
pCS68	*ada*$^+$ gene inserted in pAT153	[28]
pCS70	*ada*$^+$–*alkB*$^+$ operon inserted in pAT153	[28]

0.1 ml chloroform, the lysate was clarified by centrifugation. This method is essentially as described [29]. λ phage containing uracil in its DNA (λ-uracil) was prepared using strain CJ236 (*dut ung*), that lacks deoxyuridine triphosphatase and uracil–DNA glycosylase, and was initially infected with 1×10^5 λ_{gv} pfu.

2.3. MMS treatment of λ_{gv} phage

A λ_{gv} lysate (4×10^{11} pfu/ml) was diluted 10^2-fold in M9 minimal salts. 10 mM $MgSO_4$ and mixed with an equal volume of MMS at various concentrations in the same medium. After incubation at 37°C for 30 min, the λ phage suspension was, diluted 10^3-fold in M9 salts. 1 mM $MgSO_4$, immediately titered for survival, and then retained at 24°C for several days. Survival of the MMS-treated λ phage was titered each day for 2 more days.

2.4. Titration of λ_{gv} survival

E. coli cultures were grown in LB broth. containing 50 μg/ml carbenicillin where appropriate. to A_{600} 0.5 to 0.6. Survival of λ-uracil. or λ phage treated with MMS. was titered by plating triplicate 100 μl aliquots of various dilutions with 0.3 ml cultures in 3-ml molten soft LB agar containing 10 mM $MgSO_4$ on an LB agar. 10 mM $MgSO_4$ plate and incubating overnight at 37°C.

2.5. Monitoring for DNA strand breakage in vivo

Strains carrying plasmid pET15b (5.71 kb) were grown in M9 minimal medium pH 7 [31] supplemented with 0.2% glucose. 1 mM $MgSO_4$. 0.2% Casamino acids. 1 μg/ml thiamine hydrochloride and 50 μg/ml carbenicillin at 37°C to A_{450} 0.5. Aliquots were exposed to various doses of MMS for 20 min at 37°C. The cells were pelleted and washed. and the DNA gently extracted [32] using a QIAamp Tissue Kit (Qiagen) and analysed for open circular form by agarose gel electrophoresis followed by scanning of the ethidium bromide stained gel with a Molecular Dynamics densitometer.

2.6. Sensitivity of bacteria to $MeOSO_2$-$(CH_2)_2$-Lex and MMS

$MeOSO_2$-$(CH_2)_2$-Lex was prepared as previously described [33]. dissolved in 95% EtOH to a final concentration of 2 mM by shaking at 24°C for 40

min, then used immediately. Bacteria were cultured in supplemented M9 minimal medium pH 7 [31]. To induce the adaptive response, a non-toxic dose of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (0.5 μg/ml) was added to exponential cultures at A_{450} 0.2. Cultures at A_{450} 0.5 were exposed to various doses of $MeOSO_2$-$(CH_2)_2$-Lex for 2 h or MMS for 20 min at 37°C. After treatment, the cells were immediately diluted in M9 minimal salts and plated on LB agar plates to estimate survival.

3. Results

We first investigated the effect of an *alkB* mutation on the processing of abasic sites in vivo. Two procedures were used to form abasic sites in DNA: enzymatic excision of uracil in vivo and spontaneous depurination of MMS-treated DNA in vitro. Virulent bacteriophage λ_{gv} containing uracil in its DNA (λ-uracil) was prepared by growing λ_{gv} in strain CJ236 (*dut ung*). This strain incorporates uracil residues into DNA due to lack of dUTPase and fails to excise them because of the absence of uracil–DNA glycosylase [25]. The titre of the λ-uracil lysate on strain BD10 (*ung*) was 6.8×10^{10} pfu/ml, but decreased to 2×10^6 pfu/ml in AB1157 (a wild type strain) due to excision of uracil by uracil–DNA glycosylase and the generation of many toxic abasic sites. Survival of λ-uracil was shown to decrease a further 100-fold in strain RPC501 (*xth nfo*) that is known to be deficient in the repair of abasic sites in DNA due to the lack of two apurinic endonuclease activities, exonuclease III and endonuclease IV [34]. Survival of λ-uracil in BS87 (*alkB117*::Tn3) and HK82 (*alkB22*) was the same as that in AB1157 and HK80, their respective wild-type strains (Table 2). MMS is a weak inducer of the adaptive response to alkylation damage [3]. Resistance of a wild-type strain to MMS compared with an *alkB* mutant may therefore be enhanced due to induction of the $alkB^+$ gene. To increase the cellular level of AlkB protein in the present experiments, that is, in the absence of DNA methylation, HK82 was transformed with pCS70, a multicopy plasmid carrying the ada^+–$alkB^+$ operon. The level of Ada protein is increased by 25-fold in cells carrying this plasmid [28] and a similar increase might be expected for the AlkB protein. Lambda-uracil survival was the same in HK82/pCS70 (ada^+–$alkB^+$) as in HK82/pCS68 (ada^+) [28] (Table 2). All these observations indicate that the AlkB protein is not involved in the processing of abasic sites derived from the removal of uracil.

Table 2
Assessment of abasic site repair in *E. coli alkB* strains

E. coli strains	Relevant genotype	λ-Uracil survival (pfu/ml)[a]
BD10	*ung*	6.8×10^{10}
AB1157	Wild-type	2.0×10^6
RPC501	Δ*xth nfo*-1::Kan^r	2.2×10^4
BS87	*alkB117*::Tn3	1.5×10^6
HK80/pAT153	wild type	1.5×10^6
HK82/pAT153	*alkB22*	1.8×10^6
HK82/pCS68	*alkB22* / ada^+	2.1×10^6
KK82/pCS70	*alkB22* / ada^+ $alkB^+$	2.2×10^6

[a]λ-Uracil: λ_{gv} with DNA containing uracil was prepared by growth of λ_{gv} in CJ236 (*dut ung*) and titered on various strains.

In a second approach, λ_{gv} was treated with MMS (λ-MMS), and allowed to depurinate spontaneously at 24°C for two days in order to accumulate abasic sites derived from the loss of methylated bases. Taking into account the relative amounts of the different methylated bases formed, and their respective half-life [4], we calculate that up to 23% of the total methylated bases may depurinate during this time period. On the day of treatment (Day 0), survival of the phage treated with 50 mM or 75 mM MMS was approximately twofold greater in strain HK80 (wild type) than in HK82 (*alkB22*) (Fig. 1a). This result is in agreement with previous observations and provides evidence that *alkB* mutants are defective in processing methylated DNA [12]. Furthermore, survival of λ-MMS was greater in HK82/pCS70 (ada^+–$alkB^+$) than in HK82/pCS68 (ada^+) or HK82/pAT153 (Fig. 1b). When λ-MMS was allowed to depurinate at 24°C, survival decreased each day in both HK80 (wild-type) and HK82 (*alkB22*) by approximately two- to three-fold and four- to six-fold for 50 mM and 75 mM MMS-treated λ phage, respectively. The relative survival in the two strains remained quite constant at each time point. A similar observation was made using strains HK82/pCS70 (ada^+–$alkB^+$) compared with HK82/pCS68 (ada^-) and HK82/pAT153 (Fig. 1b).

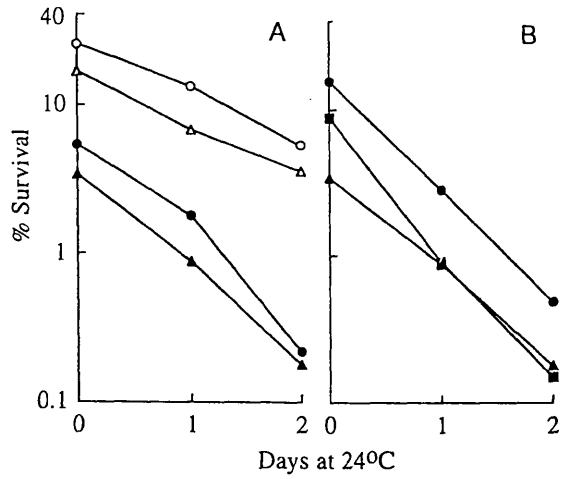

Fig. 1. (A) Survival of λ-MMS after spontaneous depurination at 24°C. λ_{gv} phage were treated with 50 mM (△,○) or 75 mM (▲,●) MMS for 30 min at 37°C, diluted and then retained at 24°C for 2 days. Survival of the λ-MMS was titered each day on strains HK80 (wild-type) (○,●) and HK82 (*alkB22*) (△,▲). (B) λ_{gv} phage was treated with 75 mM MMS, diluted and titered daily on strains HK82/pAT153 (▲), HK82/pCS68 (*ada*$^+$) (■) and HK82/pCS70 (*ada–alkB*$^+$) (●).

If the AlkB protein was involved in the repair of abasic sites, the loss of survival with time (i.e., with increasing depurination) should be greater in HK82 than in HK80 {or in HK82/pCS68 (*ada*$^+$) than in HK82/pCS70 (*ada*$^+$–*alkB*$^+$)}. This was not the case, and indicates that the AlkB protein is not required to repair abasic sites formed after MMS treatment. In addition, it suggests that AlkB protein is not required to remove secondary lesions that could arise at these sites, that is, DNA protein cross-links and DNA interstrand cross-links. This suggestion was substantiated further by extending the life of abasic sites in vivo, and therefore allowing more time for the formation of secondary lesions. Abasic sites are incised very rapidly by the apurinic endonucleases, exonuclease III (Xth) and endonuclease IV (Nfo). The life of abasic sites may therefore be extended in strains that lack these activities. Plasmids pCS70 (*ada*$^-$–*alkB*$^-$) and pCS68 (*ada*$^+$) were transformed into strains BW9109 (*xth*) and RPC501 (*xth nfo*) to determine whether increased cellular levels of AlkB protein influence survival of λ-uracil in these strains. Overexpression of the *ada–alkB* operon on pCS70 did not increase survival of λ-uracil in the AP endonuclease-deficient strains (Table 3), and is therefore a further indication that AlkB protein does not repair secondary lesions that may arise at these sites.

Direct induction of DNA strand breaks by DMS by a mechanism other than cleavage of abasic sites has been reported [22]. Such breaks are of unknown structure but are potential candidates for toxic lesions generated by this agent and possibly also by MMS. A comparison of the repair of DNA strand breaks in strains HK80 (wild-type)/pET15b and HK82 (*alkB22*)/pET15b was attempted. After MMS treatment of cells, plasmid DNA was gently extracted and analysed for open circular DNA molecules as a monitor of single strand breaks. To prevent cleavage of any abasic sites, alkaline conditions that are often used to denature chromosomal DNA and to extract plasmid DNA were avoided. No increase in the open circular form, and therefore no induction of strand breaks, was detected with doses of MMS between 0 and 60 mM, although decreased recovery of DNA (data not shown) and low survival of the *alkB* mutant occurred. MMS (25 mM) resulted in only 2% survival of HK82 (*alkB22*)/pET15b compared to 80% of HK80 (wild-type)/pET15b. Approximately 30 copies of pET15b (5.71 kb) per cell creates a plasmid target size of 1.7×10^5 base pairs compared with a chromosomal target of 4.7×10^6 base pairs. If direct strand breaks are occurring in chromosomal DNA on treatment of cells with these MMS doses, they must be rare events, and we reason that they are probably

Table 3
Influence of *alkB*$^+$ overexpression on λ-uracil survival in *E. coli* *Δxth / nfo-1* strains

E. coli strains[a]	λ-Uracil survival (pfu/ml)[b]
AB1157	1.6×10^6
BW9109/pAT153	5.6×10^5
BW9109/pCS68	5.8×10^5
BW9109/pCS70	6.5×10^5
RPC501/pAT153	1.0×10^4
RPC501/pCS68	1.1×10^4
RPC501/pCS70	1.2×10^4

[a] Strains: BW9109 (*Δxth*) and RPC501 (*Δxth nfo-1*::Kan^r). Plasmids: pAT153 (vector), pCS68 (*ada*$^+$), pCS70 (*ada-alkB*$^+$).
[b] λ-Uracil: λ_{gv} with DNA containing uracil was prepared by growth of λ_{gv} in CJ236 (*dut ung*).

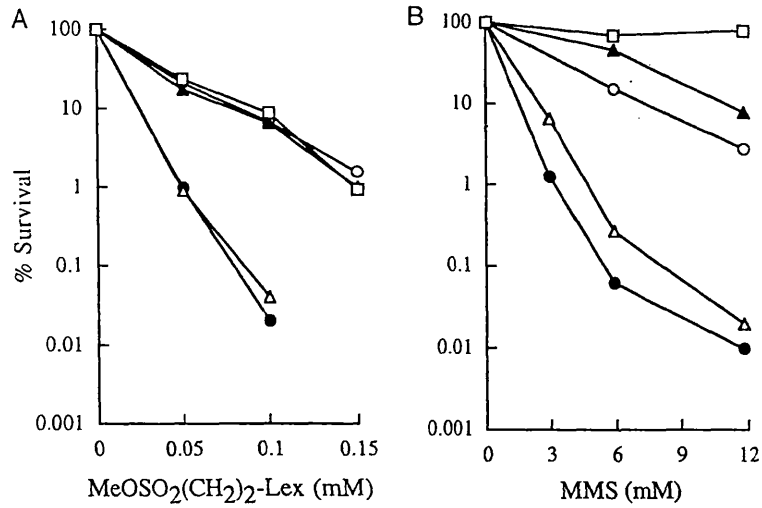

Fig. 2. Sensitivity of various strains to $MeOSO_2$-$(CH_2)_2$-Lex and MMS. Various strains were treated with increasing concentrations of (A) $MeOSO_2$-$(CH_2)_2$-Lex for 2 h or (B) MMS for 20 min, and then assayed for survival. □, AB1157 (wild type); ▲, BS87 (*alkB117*::Tn3); ○, MS23 (*alkA1*); △, GC4803 (*tagA1 alkA1*); ●, SD1 (*tagA1 alkA1 alkB117*::Tn3).

not a prime candidate for the lesion repaired by the AlkB protein.

The above experiments eliminate several types of potential toxic DNA damage as the substrate of the AlkB function. Although DNA glycosylase and DNA methyltransferase activities have not been detected previously in purified AlkB protein preparations [15]. the AlkB activity could remove a modified base by another mechanism or excise a minor DNA modification that is not readily detected. MMS induces a wide range of different modified bases in the major and minor grooves of DNA. Compounds that bind specifically to the minor groove and methylate bases only at sites within this groove have been designed. and generate fewer modifications than MMS. For example, $MeOSO_2$-$(CH_2)_2$-Lex generates primarily 3-methyladenine (a minor groove adduct) and very little 7-methylguanine (a major groove adduct) [35]. Strain GC4803 (*tagA1 alkA1*), that lacks 3-methyladenine DNA glycosylases I and II and is therefore deficient in repair of 3-methyladenine in DNA. was very sensitive to this compound (Fig. 2A). To examine whether AlkB protein repairs a modified base in the minor groove of DNA. an *alkB* mutant was monitored for sensitivity to $MeOSO_2$-$(CH_2)_2$-Lex. Neither strain BS87 (*alkB117*::Tn3) nor MS23 (*alkA1*) were sensitive to $MeOSO_2$-$(CH_2)_2$-Lex (Fig. 2A). although as expected, they were both sensitive to MMS (Fig. 2B). The same result was obtained even when the cells were preexposed to a low nontoxic dose of MNNG to increase expression of the *alkA* and *alkB* genes in the wild-type strain (data not shown). A triple mutant SD1 (*tagA1 alkA1 alkB*) had a similar sensitivity as GC4803 (*tagA1 alkA1*) to $MeOSO_2$-$(CH_2)_2$-Lex but appeared to be slightly more sensitive to MMS (Fig. 2). These observations indicate that AlkB protein repairs a different lesion to the Tag protein that is generated by MMS but not by $MeOSO_2$-$(CH_2)_2$-Lex, and verifies that AlkB protein does not repair 3-methyladenine.

4. Discussion

The function of the *E. coli* AlkB protein in the processing of DNA damage generated by MMS is undefined. Here, we have considered several types of DNA lesions, that are known to be generated by MMS. as possible substrates of the AlkB function. All the lesions considered would block DNA replication if unrepaired and therefore result in cell death. They include apyrimidinic sites (formed by excision of uracil from DNA in vivo) and apurinic sites (formed by spontaneous depurination of MMS-treated phage DNA in vitro), secondary lesions that could

arise at either of these sites (DNA–protein cross-links and DNA interstrand cross-links), and 3-methyladenine. We have found no defect in the processing of any of these DNA lesions in the *alkB* mutant.

Lambda phage treated with MMS has a lower survival in an *alkB* mutant compared to the wild-type strain. This observation indicates that the DNA damage acted upon by AlkB protein can be generated in vitro as well as in vivo. Nevertheless, the possibility that the particular lesion repaired by AlkB is generated more readily in vivo, due to modification of MMS by a cellular metabolite(s), could explain several observations: the extreme sensitivity of *alkB* mutants to MMS but only a small decrease in λ-MMS survival in such strains [12]; the failure so far to detect any activity of purified AlkB protein when using DNA methylated by MMS in vitro as the substrate [15]; and possibly the suppression by several unrelated yeast gene products of the sensitivity of *alkB*, but not *tag alkA* and *recA* mutants, to MMS [36]. The difference in λ-MMS survival in the wild type and the *alkB* mutant remained constant when the λ-MMS was retained at 24°C for 2 days showing that the lesion acted upon by the AlkB protein was stable in these conditions. Thus, for example, it is not a modified base that readily depurinates, and also it does not arise from spontaneous conversion of one type of damage into another as occurs, for example, when the imidazole ring of 7-methylguanine opens to form a formamidopyrimidine.

We have eliminated several MMS-induced DNA lesions as substrates of the AlkB protein. Other possible candidates could be minor modified bases induced by MMS or previously uncharacterised lesions that are not separated from the major methylation products using documented HPLC procedures. Both the Tag and AlkA proteins remove 3-methyladenine from DNA. The AlkA protein has a wider substrate range than Tag and also excises several minor adducts [9,10,37], for example 3-methylguanine, which constitutes only 0.6% of the total methylation products in MMS-treated DNA [4]. Lack of sensitivity of an *alkA* mutant to $MeOSO_2$-$(CH_2)_2$-Lex indicates that the Tag protein was sufficient to repair all the 3-methyladenine formed in DNA and that 3-methylguanine, that is inefficiently repaired by Tag [10], was not generated in sufficient amounts to result in significant toxicity. In support of this suggestion, the ratio of 3-methylguanine to 3-methyladenine was found to be 1 to 30 in MMS-treated DNA but only 1 to 400 for $MeOSO_2$-$(CH_2)_2$-Lex treated DNA (B.G. and J. Kelly, unpublished data). Low generation of 3-methylguanine lesions by $MeOSO_2$-$(CH_2)_2$-Lex may be due to its preferential binding to A/T rich sequences, therefore directing this compound to particularly alkylate adenines in the minor groove of DNA [35]. $MeOSO_2$-$(CH_2)_2$-Lex may therefore serve as a powerful tool to characterise alkylation-sensitive mutants for a specific defect in the repair of 3-methyladenine in DNA. The observation that *alkA tag* and *alkA tag alkB* mutants had a similar sensitivity to $MeOSO_2$-$(CH_2)_2$-Lex suggests that 3-methyladenine is not a substrate of the AlkB protein but does not eliminate other modified bases that could be induced by MMS in the minor or major groove of DNA.

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