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The construction of several nitroreductase and *O*-acetyltransferase overproducing *S. typhimurium* tester strains and their application in the *Salmonella* mutagenicity assay (Ames test)

by

Craig E. Carroll, B. Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

Ottawa, Canada

2000

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**The construction of several nitroreductase and *O*-acetyltransferase overproducing *S.*
typhimurium tester strains and their application in the *Salmonella* mutagenicity assay
(Ames test)**

Submitted by

Craig E. Carroll B. Sc. (Hons.)

**in partial fulfilment of the requirements
for the degree of Master of Science**

Chair, Department of Biology

Thesis Supervisor

Carleton University

Abstract

The major (*nfsA*) and minor (*nfsB*) *Escherichia coli* nitroreductase genes are involved in the metabolic activation of nitrosubstituted compounds. Plasmids harbouring cloned nitroreductase genes in different combinations with *Salmonella typhimurium* OAT and *E. coli mucA/B* were introduced into *S. typhimurium* mutagenicity tester strains TA1535, and TA1538. High levels of nitrofurazone reductase activity was observed in strains expressing plasmid-borne nitroreductases. Several of the resulting strains were extremely sensitive toward some nitrosubstituted compounds. Mutagenicity testing with nitrosubstituted compounds revealed that NfsA preferentially induces both frameshift and base substitution mutations for compounds such as 2-NF, 1,6-DNP, and 2-NN whereas NfsB only stimulates 1-NP mutagenesis and may inhibit the mutagenicity of some nitrosubstituted chemicals. The results indicate that some of the newly constructed *S. typhimurium* tester strains have a broader substrate specificity, and are more sensitive than the previously established strains.

Acknowledgments

I would like to thank my supervisor Dr. I. B. Lambert for providing me with the opportunity to do my Master's research in his laboratory at Carleton University. I thank him also for providing me with the opportunity to work as a laboratory technician in his lab while pursuing my studies at Carleton. I thank all the members of the Lambert-Smith lab who have helped and provide valuable insight into various aspects of this project. Their advice and friendship helped make the Lambert-Smith lab an enjoyable place to work.

I wish to thank the members of my advisory committee Dr. J. Vierula and Dr. D. Gould for their patience and advice. I also would like to thank Dr. G. Carmody for his valuable assistance with the statistical aspects of this work.

I am grateful to Dinesh Warnakulasuriyarachchi who helped with the Ames testing. His hard work and dedication was really appreciated at a time when I needed to get the experiments completed in a timely fashion.

My special thanks go to my good friend Adam Baker. His friendship and advice throughout my years at Carleton was much appreciated. I am also grateful to him for introducing me to my girlfriend Andrea Rowan. Andrea has helped me in many ways throughout my Master's and I am grateful to her for her patience, constant encouragement, support, and love.

Last but not least, I am indebted to my parents who have guided me and always supported me over the years. Their love and encouragement and help putting this thesis together while I was living in Saskatchewan was much appreciated.

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List of Abbreviations

A	adenine
Amp	ampicillin
ATP	adenosine triphosphate
BDF	bioassay-directed fractionation
<i>bio</i>	biotin mutation
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
°C	temperature in degree Celsius
CFU/ml	colony forming units/milliliter
<i>cnr</i>	the minor nitroreductase from <i>S. typhimurium</i>
Cys⁶⁹	cysteine residue 69
ddH₂O	deionized, distilled water
1,6-DNP	1,6-dinitropyrene
1,8-DNP	1,8-dinitropyrene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetra-acetate
EMS	ethyl methanesulfonate

EtBr	ethidium bromide
ETOHNaC	ethanol sodium acetate
FMN	flavin mononucleotide
Frp	a flavin oxidoreductase from <i>Vibrio harveyi</i>
G	guanine
<i>gal</i>	galactose mutation
<i>hisC</i>	a structural gene in the histidine operon
<i>hisD</i>	histidinol dehydrogenase gene
<i>hisD3052</i>	a mutation in the histidinol dehydrogenase gene
<i>hisG</i>	first structural gene in the histidine operon
<i>hisG46</i>	a mutation in the first gene encoding the first enzyme of histidine biosynthesis
ICR-191	(2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine 2HCl
kb	kilobase(s)
kDa	kilodaltons
Km	kanamycin
kV	kilovolts
<i>lacI</i>	gene encoding the <i>lac</i> operon repressor
<i>lacZ</i>	gene encoding the enzyme β-galactosidase
LB	Luria-Bertani
LPS	lipopolysaccharide
M	moles/liter
m⁺_k	methylation positive <i>E. coli</i> K-12 strain

M13	a type of bacteriophage that produces ssDNA
μg	microgram(s)
μl	microliter(s)
μmol	micromole(s)
μMol	micromoles/liter
mg	milligram(s)
ml	milliliter(s)
mM	millimolar
<i>mucA/B</i>	replicational accessory proteins
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
<i>NAT1</i>	human NAT1 gene
<i>NAT2</i>	human NAT2 gene
NF	nitrofurazone
NFR	nitrofurazone reductase
<i>nfsA</i>	The major nitroreductase in <i>E. coli</i>
<i>nfsB</i>	The minor nitroreductase in <i>E. coli</i>
ng	nanogram(s)
nitro-PAH	nitrated polycyclic aromatic hydrocarbon
1,6-NONP	1-nitroso-6-nitropyrene
1,8-NONP	1-nitroso-8-nitropyrene
1-NP	1-nitropyrene

2-NF	2-nitrofluorene
2-NN	2-nitronaphthalene
4-NQO	4-nitroquinoline-1-oxide
nmole	nanomole
NR	nitroreductase activity
O.D.	optical density
<i>OAT</i>	O-acetyltransferase
ONB#2	Oxoid nutrient broth #2
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
%	percent
pmole	picomole
r_k	restriction deficient <i>E. coli</i> K-12 strain
<i>rfa</i>	deep rough mutation
RNA	ribonucleic acid
RNase	an enzyme that digests RNA
RPM	revolutions per minute
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
S.D.	standard deviation
SDS	sodium dodecyl sulphate
<i>snrA</i>	The major nitroreductase in <i>S. typhimurium</i>
SOS	an emergency response system in <i>E. coli</i> that is induced upon DNA damage

SSB	<i>E. coli</i> single stranded binding protein
SSC	sodium, sodium citrate solution
ssDNA	single stranded DNA
SSPE	sodium, sodium phosphate, EDTA solution
STT	short-term test
T	thymine
TAE	tris-acetate, EDTA buffer
TE	tris-HCl, EDTA buffer
Tet	tetracycline
UV	ultraviolet
UVR ABC	DNA excision repair system
<i>ΔuvrB</i>	a mutation in the <i>uvrB</i> gene of the uvrABC exinuclease complex
V	volt(s)
VB	Vogel and Bonner salts
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Toxicology testing

Human exposure to potentially harmful chemicals in the environment and workplace is a serious health concern. There is a constant input of these chemicals into our environment by industries, through the combustion of fossil fuels, and through various waste disposal procedures. Many of these chemicals have the potential to damage cellular DNA and cause mutations which may lead to diseases such as cancer (Sullivan *et al.*, 1993). In fact, there is a growing list of environmental pollutants which have been linked to cancer. Included in this list are a class of compounds known as polycyclic aromatic hydrocarbons (PAHs) and their nitrated derivatives (nitro-PAHs) which are the subject of study in this thesis. Both PAHs and nitro-PAHs have been detected in the earth's atmosphere, soil, water, and in cooked foods (Rosenkranz and Mermelstein, 1983; Josephy *et al.*, 1997). Several of these compounds are potent bacterial mutagens, mammalian cell mutagens, clastogens, and some are rodent carcinogens (Mullin *et al.*, 1987; Rosenkranz and Mermelstein, 1983).

Chemicals interact with DNA in several different ways, and doing so, cause specific types of DNA damage that are characteristic of the chemical. The type of induced DNA damage and cellular mechanisms that deal with this damage, often play a key role in determining what class of mutation will result. One type of DNA damage that has received significant attention over the past 30 years is the formation of DNA adducts. A DNA adduct is formed when a chemical, or a metabolically activated derivative, reacts with specific atoms in DNA. Several diseases, including cancer, are now being linked to the ability of chemicals to form DNA adducts. Moreover, DNA adduct formation is now accepted as a common attribute of most potent carcinogens (Dipple, 1995).

In many countries, regulatory guidelines require biological testing of new compounds that are intended for human use, or for release into the environment. There is a need for sensitive toxicological tests which do not involve the use of animals. Often the first type of biological testing performed is a short-term test (STT). Currently, there are over 200 STTs available for the detection and identification of genotoxic compounds (DeMarini *et al.*, 1989). The four most commonly used STTs are the Ames *Salmonella his⁻* reversion assay, the chromosome aberration assay, sister chromatid exchange induction in Chinese hamster ovary cells, and the mouse lymphoma L5178Y cell mutagenesis assay (Tennant *et al.*, 1987). STTs offer several advantages when compared to long-term carcinogenicity tests with rodents: they are quick, sensitive, dependable, relatively inexpensive, and do not involve treatment of laboratory animals (Tennant *et al.*, 1987).

Since the role of somatic mutations in carcinogenesis has been firmly established, the role STTs has played in testing has dramatically increased. Much effort has been put into developing a STT that can predict rodent carcinogenicity with a high degree of certainty. Early studies showed that several STTs, including the Ames *Salmonella* mutagenesis assay, were a good predictor of rodent carcinogenicity (Tennant *et al.*, 1987). However, after further evaluation, it became apparent that the Ames test alone was not an adequate predictor of rodent carcinogenicity for certain classes of chemicals (Fetterman *et al.*, 1997; DeMarini *et al.*, 1989). Subsequently, a battery of STTs was thought to be a more effective approach for predicting rodent carcinogenicity because the limitations of one test could be compensated for by another test. However, based on concordance data against mouse and rat carcinogens, a single STT was found to perform

as well as a battery of STTs (DeMarini *et al.*, 1989). Compounds that are inaccurately predicted in STTs are of two types: false positives and false negatives. A false positive is a chemical which exhibits a positive genotoxic response, but is negative in rodent carcinogenicity assays. Conversely, a false negative is a chemical which gives a negative genotoxic response, but is positive in rodent carcinogenicity assays (DeMarini *et al.* 1989). Despite the limitations of STTs, they offer several advantages and are still routinely used to detect genotoxic agents (DeMarini *et al.*, 1989).

1.1.1 Prokaryotic test systems

For more than two decades, prokaryotic mutagenicity assays have been used by research laboratories and regulatory agencies around the world to 1) identify potential genotoxic chemicals; 2) assess the potential health risks associated with environmental chemical exposure; and 3) provide insight into the biochemical mechanisms of mutagenesis (Josephy *et al.*, 1997). The idea of a bacterial assay that could detect the mutagenicity of potential carcinogens has been an attractive one due to their speed, reliability, and cost effectiveness. The earliest bacterial mutagenicity assays were forward or reversion assays. A forward mutagenicity assay detects mutations in a target gene that results in the loss of function of the corresponding protein, such as the *lacI* or *lacZ* gene products of the *Escherichia coli* *lac* operon (Miller, 1992). Conversely, a reversion assay detects mutations in a target gene that causes the gain of function of a particular gene product, such as histidine dehydrogenase (*hisD* gene) in *Salmonella typhimurium* (Hartman *et al.*, 1986).

1.1.2 Ames test (*Salmonella typhimurium* *his*⁻ reversion assay)

By far the most commonly used STT is the Ames test (*Salmonella typhimurium* *his*⁻ reversion assay) developed by Bruce Ames and colleagues in the early 1970's (Ames *et al.*, 1973; Ames *et al.*, 1975). Over 5000 compounds have been catalogued using this methodology and it is still used today by regulatory agencies and research laboratories world-wide (Josephy *et al.*, 1997). The Ames test is primarily used as a preliminary screen during chemical and drug development. Depending on the outcome, researchers can evaluate the genotoxic potential of a compound, and either halt development or pursue further toxicological testing in mammalian systems (Fetterman *et al.*, 1997). In addition, the Ames test has had tremendous success detecting genotoxic agents in complex environmental mixtures. This approach involves chemically fractionating the mixture and testing each fraction with the Ames test, a technique called bioassay-directed fractionation (Legzdins *et al.*, 1995). Besides providing genotoxicity information, the Ames test also provides data regarding both mutational specificity (i.e. frameshift versus base substitution mutations) and site specificity (i.e. -AAAAAAA- versus -CCCCCC-).

By itself, the Ames test is limited to testing only direct acting mutagens. Since the majority of carcinogens are converted or 'bioactivated' to DNA-reactive species by mammalian enzymes, this represents a significant limitation to the Ames test (Josephy *et al.*, 1997). To overcome this drawback, an homogenate called S9, prepared from rodent liver, was used in the Ames test to provide necessary enzymes for the bioactivation of potential carcinogens (Maron and Ames, 1983).

The Ames test is a bacterial reversion assay which scores reverse mutations in several genes, *hisD*, *hisG*, and *hisC*, that are located in the histidine operon (Hartman *et*

al., 1986). The assay involves treating *S. typhimurium* histidine dependent strains with a chemical or complex mixture. Following a specific mutagenic event, the tester strains revert to a histidine independent phenotype (Ames *et al.*, 1973). Revertants are selected on minimal glucose medium supplemented with trace amounts of histidine to allow limited growth of all cells. However, only the cells which have reverted can continue to grow and form colonies (see Fig. 1) (Maron and Ames, 1983). There are several different standard Ames tester strains available; each one contains a different *his*⁻ mutation and detects a specific type of mutation. All strains were originally derived from *S. typhimurium* LT2 and have been further modified to enhance the sensitivity of the strain (Ames *et al.*, 1973). One modification (*rfa*) causes partial loss of the lipopolysaccharide (LPS) barrier down to the ketodeoxyoctanoate-lipid core, making the bacterium much more permeable to large molecules that under normal conditions would not penetrate the cell wall (Ames *et al.*, 1973). Another modification is a deletion in the *uvrB* gene that renders the bacterium deficient in DNA nucleotide excision repair system (UVR ABC exonuclease), the primary mechanism for removal of bulky DNA adducts (Maron and Ames, 1983). The introduction of the R-factor plasmid, pKM101 to several tester strains also enhances the sensitivity of the assay towards many chemicals by increasing the frequency of lesion bypass resulting in enhanced mutagenesis and survival (Walker, 1984; Bridges and Woodgate, 1985).

There are several *S. typhimurium* standard tester strains that are recommended for general mutagenicity testing, TA97, TA98, TA100, and TA102 (see Table 1). Each strain carries a different mutation in the histidine operon, and reverts following specific types of mutation. For instance, TA1535 is a base substitution strain and with the

Figure 1. A schematic representation of the mutagenicity assay. Following an overnight growth period of 12 hours, an aliquot of the tester strain is mixed with molten top agar containing trace amounts of histidine and biotin. An aliquot of the chemical being tested is added to the top agar, mixed, and poured onto a minimal agar plate. The negative control receives only the solvent used to dissolve the chemical. The plates are incubated for 48 hours at 37 °C and counted using an AccuCount 1000 automatic colony counter. The number of induced revertants per plate are calculated by subtracting the number of spontaneous revertants. The final result is expressed as the number of induced revertants per nanomole of the test chemical.

S. typhimurium Reversion Assay

tester strain O/N

mutagen

Table 1. Genotypes of several conventional *S. typhimurium* tester strains. The strains were originally derived from *S. typhimurium* LT2. The strains used routinely in mutagenicity testing are TA100 and TA98. The *rfa* mutation increases the permeability of the cell wall. The $\Delta uvrB$ mutation abolished the bacterial UVRABC excision nuclease. The R-factor is pKM101 and the Δgal mutation prevents the synthesis of galactose (Adapted from Maron and Ames, 1983).

Histidine mutation				LPS	Repair	R-factor
hisD6610 hisO1242 = TA88	hisD3052	hisG46	hisG428 (pAQ1)			
TA90	TA1538	TA1535	-	<i>rfa</i>	$\Delta uvrB$	- R
[TA97]	[TA98]	[TA100]	-	<i>rfa</i>	$\Delta uvrB$	+ R
-	TA1978	TA1975	-	<i>rfa</i>	+	- R
TA110	TA94	TA92	-	+	+	+ R
-	TA1534	TA1950	-	+	$\Delta uvrB$	- R
-	-	TA2410	-	+	$\Delta uvrB$	+ R
TA89	TA1964	TA1530	-	Δgal	$\Delta uvrB$	- R
-	TA2641	TA2631	-	Δgal	$\Delta uvrB$	+ R
-	-	-	[TA102]	<i>rfa</i>	+	+ R

introduction of pKM101 becomes TA100 (see Table 1) (Maron and Ames, 1983). TA1535 and strains derived from it harbour the *hisG46* mutation, a mutation in the first structural gene of the histidine operon (Hartman *et al.*, 1986).

The *hisG46* mutation is an A·T→G·C transition that substitutes a proline residue (-CCC-) for a leucine (-CTC-) in the wild-type organism (see Fig. 2) (Hartman *et al.*, 1986). Nearly all possible base substitution mutations occurring at one of the G-C base pairs in the target sequence will revert TA1535 and its derivatives. In addition, an extragenic tRNA suppressor mutation can also cause a reversion to occur (Hartman *et al.*, 1986).

The frameshift strain TA1538 and its R-factor derivative TA98 (see Table 1) contain the *hisD3052* mutation situated in the histidinol dehydrogenase gene (*hisD* gene) (Hartman *et al.*, 1986). The mutational target sequence is a sequence of eight repetitive (-GC-) residues which is situated upstream from a -(C) frameshift mutation (see Fig 3) (Hartman *et al.*, 1986). Since the target sequence is different than the *his^r* mutation, reversion at the *hisD3052* locus is not a true reversion.

Streisinger *et al.* (1966) proposed that during DNA replication, misalignment often occurs in repetitive sequences creating a bulge of one or more extrahelical nucleotides. The misalignment is stabilized by normal base pairing of the flanking repetitive nucleotides and extension from this terminus leads to frameshift mutations (Streisinger *et al.*, 1966). The *hisD3052* mutational target sequence is a known hotspot for -2 frameshifts and will revert upon a -(GC) or -(CG) mutational event (Maron and Ames, 1983). In addition, due to the nature of the target sequence, many different

Figure 2. Mutational target sequence for reversion of base substitution *hisG46* in *S. typhimurium* TA1535. Note that the mutational target sequence is the same as the histidine mutation that renders the bacterium dependent on histidine for growth. Several of the possible G:C -> T:A, G:C -> A:T and G:C -> C:G changes occurring at *hisG46* will cause a reversion. In addition, an extragenic tRNA suppressor mutation (A:T -> C:G) can cause "reversion" of the *hisG46* mutation (Adapted from Hartman *et al.*, 1986).

Figure 3. Mutational target sequence for reversion of frameshift *hisD3052* in *S. typhimurium* TA1538. The primary mutational target sequence (a 5'-CGCGCGCG-3' sequence at which -(CG) mutations can be scored) is situated upstream from the *hisD3052* mutation which is a (-C) mutation that renders the bacterium dependent on histidine for growth. Many different types of frameshifts can cause a reversion provided they restore the correct reading frame. These mutations may occur at the “reversion site” itself or may be compensatory mutations at other sites (Adapted from Hartman *et al.*, 1986).

Wild Type
Gln Leu Ala Glu Leu Pro Arg Ala Asp Thr Ala Arg Gln Ala Leu Ser Ala....
CAA CTG GCG GAA CTG CCG CGC GCG GAC ACC GGC CGG CAG GCC CTG AGC GCC

ICR364-OH -1 bp

ms03052 CAA CTG GCG GAA CTG CCG CGC GCG GAC ACC GGC GGC AGG CCC TGA
nonsense
+1 bp ICR, NG

spont.,
MF, NO, MC
NG, ICR
(-CG or -GC)
-2bp

CTG CCG CGC GGA CAC CGC CGG CAG GCC CTG
Leu Pro Arg Gly His Arg Arg Gln Ala Leu....

sequence alterations spanning a region of more than 50 base pairs can cause a reversion provided they restore the correct reading frame (Josephy *et al.*, 1997).

A typical Ames test would include TA100, TA98, and possibly other strains developed by the Ames laboratory that are not discussed here (see Table 1). The strains used for toxicology testing often depend on the class of chemical being tested. Due to its versatility and cost-effectiveness, the Ames test is extremely well validated and has been adopted by many government and regulatory agencies such as the National Toxicology Program (NTP), and the National Cancer Institute (NCI).

1.1.3 *LacZ* reversion mutagenicity assay

Several other mutagenicity assays have been developed with various bacterial strains to detect chemical mutagens (Ames *et al.*, 1973). One such system utilizes several engineered *E. coli* strains that have specific mutations in the *lacZ* gene or β -galactosidase gene product (Cupples and Miller, 1989; Cupples *et al.*, 1990). There are eleven strains, 6 base substitution strains, and 5 frameshift strains; all having the phenotype *lacI⁻Z⁻*. Each strain will revert to *lac⁺* upon a specific type of mutational event which is indicated by the formation of a blue colony on X-Gal (5-bromo-4-chloro-3-indolyl- β -galactosidase) media (Cupples *et al.*, 1990). The Ames test and *lacZ* test in *E. coli* share many similarities. However, there is one distinct difference, the *lacZ* mutagenicity assay provides more mutational specificity information due to the fact that the strains are designed to revert following one specific mutation at a single site, whereas in *S. typhimurium* tester strain TA1535, for example, any one of 5 possible base substitutions and extragenic suppressors can cause a reversion. Similarly, in the

frameshift strain TA1538, all sorts of frameshifts of the -8, -5, +1, and +4 types that occur at the target site, or flanking sequences, may cause a reversion.

1.1.4 *lacI* forward mutagenicity assay

Sophisticated forward mutational systems are currently available which facilitate genetic characterization, cloning, mapping, and DNA sequence analysis (Lambert *et al.*, 1991a; Lambert *et al.*, 1992; Bertenyi and Lambert, 1996; Lambert *et al.*, 1998). In contrast to reversion assays, forward mutation assays detect mutations in a target gene that cause a loss of function in the corresponding protein. However, forward mutational systems are generally more informative than reversion assays because a large number of sites can be monitored simultaneously generating a complete mutational spectrum. One such system utilizes the *lac* repressor (*lacI*) gene in *E. coli*. The use of the *lacI* system for mutational studies was initially developed by Jeffrey Miller (reviewed in Miller, 1983) and further modified by Schaaper *et al.* (1985). The *lacI* gene is extremely well characterized and provides a rich mutational target >1000 bp for mutagenic studies (Miller, 1978). As a mutational system, the *lacI* gene is extremely sensitive to nonsense and frameshifts mutations along ~90% of the gene (Calos and Miller, 1981; Lambert *et al.*, 1992). Other mutations such as deletions and insertions can be detected; however, missense mutations are unevenly dispersed throughout the gene (Gordon *et al.*, 1988).

1.2 Nitrosubstituted compounds

Nitrosubstituted compounds are a large group of chemicals which have received significant attention over the past 30 years. Many of these compounds are potent

bacterial mutagens, mammalian cell mutagens, clastogens, and some are rodent carcinogens (Mullin *et al.*, 1987; Rosenkranz and Mermelstein, 1983). Their extensive use in commercial industries and their persistence in the environment, make them a potential human health hazard. For this reason, the mutagenicity of nitrosubstituted compounds has been assessed using many STTs including the Ames test (Rosenkranz and Mermelstein, 1983). There are three classes of nitrosubstituted compounds: conjugated non-fused nitroaromatic compounds (Fig. 4I), nitrosubstituted polycyclic aromatic compounds (nitroarenes) (Figs. 4A, B, C, D, E, and G), and nitroheterocyclic aromatic compounds such as nitrofurans, nitroimidazole, and nitrothiazoles (Figs. 4F and H).

The non-fused compounds pose an occupational risk as they are used extensively in the manufacturing of polyurethanes, rubber processing chemicals, pesticides, dyes, pharmaceuticals, and explosives. Ames reversion assays conducted with non-fused nitroaromatic compounds report mutagenicities that rarely exceed 1000 revertants/nanomole (Mullin *et al.*, 1987). In terms of mutagenicity, non-fused compounds are substantially lower than the nitrosubstituted polycyclic compounds; however, they may still pose a human health risk and should be used with caution.

To date, the most mutagenic of all nitrosubstituted compounds are a class of environmental pollutants known as nitroarenes. Nitroarenes are a group of planar, fused ring, aromatic hydrocarbons containing one or more nitro moieties covalently linked to cyclic carbon atoms (see Fig. 4). Their formation stems from atmospheric reactions of PAHs, oxides of nitrogen, and traces of acid (Pitts *et al.*, 1978), and from incomplete combustion processes (Rosenkranz and Mermelstein, 1983). At least 200 nitroarenes have been identified, some of which have been detected in exhaust emissions from jet,

Figure 4. Chemical structures of several nitro-substituted compounds. Nitrosubstituted polycyclic aromatic compounds (nitroarenes) represented by A (1,8-dinitropyrene), B (1,6-dinitropyrene), C (1-nitropyrene), D (2-nitronaphthalene), E (2-nitrofluorene), and G (4-nitroquinoline N-oxide). Nitroheterocyclic compounds represented by F (nitrofurazone) and H (AF2); conjugated non-fused nitroaromatic compounds represented by I (nitrostilbene).

(A) 1,8-dinitropyrene
(1,8-DNP)

(B) 1,6-dinitropyrene
(1,6-DNP)

(C) 1-nitropyrene
(1-NP)

(D) 2-nitronaphthalene
(2-NN)

(E) 2-nitrofluorene
(2-NF)

(F) nitrofurazone
(NF)

(G) 4-nitroquinoline *N*-oxide
(4-NQO)

(H) 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide
(AF2)

(I) 4-nitrostilbene

diesel, and gasoline engines (Rosenkranz, 1982; Rosenkranz, 1996; Schuetzle *et al.*, 1982), exhausts from kerosene heaters (Möller *et al.*, 1993), urban air particulates (Tokiwa *et al.*, 1983; Arey *et al.*, 1988; Hayakawa *et al.*, 1995), river sediments (Rosenkranz and Howard, 1986), cigarette smoke condensates, incense smoke condensates (McCoy and Rosenkranz, 1982; El-Bayoumy *et al.*, 1985; Chen and Lee, 1996), the carbon blacks of photocopier toners used prior to 1979 (Löfroth *et al.*, 1980; Rosenkranz *et al.*, 1980), and grilled chicken (Kinouchi *et al.*, 1986). The most significant source of nitroarenes is the particulate emissions from diesel engines (Tokiwa and Ohnishi, 1986). Among the nitroarenes, the dinitropyrenes (DNPs) (see Figs. 4A and B) are the most mutagenic and best characterized. 1,8-dinitropyrene (1,8-DNP) and other nitropyrenes are potent frameshift mutagens and are proven rodent carcinogens (Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). For example a standard Ames test with 1,8 dinitropyrene typically yields 250, 000 revertants/nanomole in the *S typhimurium* tester strain TA98 (Tokiwa and Ohnishi, 1986). This makes 1,8-DNP among the most mutagenic compounds known. Generally, the mutation type and, to a lesser degree, the potency of the compound is related to the number of fused aromatic rings. For instance, bicyclic arenes such as 2-nitronaphthalene generally induce base substitutions, whereas nitroarenes such as NPs, DNPs, and 2-nitrofluorene (2-NF) with three or more aromatic rings usually induce frameshift mutations (Tokiwa and Ohnishi, 1986). Moreover, compounds with 4 or more rings have reduced mutagenicity.

The nitroheterocyclic aromatic compounds such as the nitrofurans (see Figs. 4F and H) have been used worldwide as human and veterinary medicines, food preservatives, and feed additives (Ni *et al.*, 1987). Many of these compounds are

mutagenic in bacteria and have caused tumors in laboratory animals (McCalla, 1983). In Ames tests, the mutagenicities reported for this group of compounds is exceedingly low when compared to the dinitropyrenes. A typical nitrofur such as nitrofurazone, (see Fig. 4F) yields 396 revertants/nanomole in TA100 and 5 revertants/nanomole in TA98 (Hagiwara *et al.*, 1993; Einistö *et al.*, 1991). Although some nitrofurans are bacterial mutagens, they continue to be used as antimicrobial agents around the world (reviewed in McCalla, 1983).

1.3 Metabolic and mutagenic functions

Most genotoxins including nitrosubstituted compounds, are not direct acting. Moreover, their mutagenicity depends on intra-and/or extra cellular metabolic activation or bioactivation from enzymes present in both prokaryotic and eukaryotic cells (Josephy *et al.*, 1997). Bioactivation produces electrophilic species which can covalently bind to DNA and proteins (King, 1988). In bacteria and mammals, the mutagenicity of nitroaromatic compounds is dependent upon the biological reduction of a single NO₂ functional group by enzymes collectively known as nitroreductases (Rosenkranz and Howard, 1986; Norman *et al.*, 1990). The intermediates of nitroaromatic reduction are *N*-hydroxyarylamine intermediates which can either spontaneously hydrolyze or undergo *O*-acetylation to form the penultimate mutagen, *N*-acetoxy-arylamine (see Fig. 5). The enzyme responsible for acetylation of the *N*-hydroxyarylamine intermediate is *O*-acetyltransferase (OAT) (Watanabe *et al.*, 1992). The *N*-acetoxy-arylamine intermediate is unstable; cleavage of the N-O bond creates nitrenium ions which are highly reactive, and capable of covalently binding to nucleic acids, forming DNA adducts (Rosenkranz

Figure 5. The proposed pathway for the metabolic activation of 1,6-dinitropyrene (1,6-DNP) by bacterial oxygen-insensitive (Type I) nitroreductases. The first activated intermediate is 1-nitroso-6-nitropyrene, which is further reduced to *N*-hydroxyl-1-amino-6-nitropyrene. Following *O* -acetylation, the *N*-acetoxy-arylamine goes on to form a nitrenium ion which can then react with DNA. Protonation of the hydroxylamine derivative can also produce the nitrenium ion. The ultimate mutagen is the guanine C(8) DNA adduct (Adapted from Rosenkranz and Howard, 1986).

and Howard, 1986). One of the best characterized DNA adducts is the guanine C(8) adduct (see Fig. 6) formed by the nitrenium ion derived from reductive metabolism of 1,8-DNP (Andrews *et al.*, 1986; Lambert *et al.*, 1991a). Although it is unclear whether this adduct disrupts hydrogen bonding in the DNA duplex, it does however impede normal DNA replication and has mutagenic consequences.

The biological activity of nitrofurans is also dependent on the reduction of a nitro moiety by nitroreductases (see Fig. 7) (McCalla, 1983; Bertenyi and Lambert, 1996). There are two potential pathways: a one electron pathway and a two electron pathway. The one electron pathway involves the transfer of one electron by a nitroreductase to the NO₂ group. In the presence of oxygen, the NO₂ group becomes trapped in a futile cycle (Whiteway *et al.*, 1998). Reductive metabolism of nitrofurans via the two electron pathway involves the transfer of two electrons to the NO₂ group to yield a nitroso-derivative and another two electron transfer to yield a *N*-hydroxylamine intermediate. The hydroxylamine intermediate can either form a stable 5-aminofuran derivative or rearrange to form an open-chain acrylonitrile derivative (McCalla, 1983). The acrylonitrile derivative is unstable and can form an open-chain nitrile which is biologically inactive. In addition, it has been suggested that the acrylonitrile derivative can interact with DNA with mutational consequences (Bertenyi and Lambert, 1996). DNA adducts formed from the reductive metabolism of nitrofurans are too labile and still remain uncharacterized.

Figure 6. The ultimate mutagen of 1,6-dinitropyrene; N-(2'-deoxyguanosin-8-yl)-1-amino-6-nitropyrene [dG-C(8)-A6NP].

Figure 7. The proposed pathway for the metabolic activation of nitrofurans by bacterial oxygen-insensitive (Type I) and oxygen-sensitive (Type II) nitroreductases. In the presence of oxygen, the 5-nitrofurans become trapped in a futile cycle. In the absence of oxygen, the 5-nitrofurans get converted into either an open-chain nitrile isomer or an aminofuran derivative, both of which are both biologically inactive. However, open-chain acrylonitrile and the nitrenium ions are reactive intermediates formed in the metabolic pathways leading to open-chain nitriles and aminofurans, respectively, and are postulated to form DNA adducts (Adapted from Bertenyi and Lambert, 1996).

1.3.1 Nitroreductases

Reduction of the NO₂ group by nitroreductase is the first and the most important step in the reductive metabolism of nitroaromatics and nitroheterocyclics. In *E. coli*, two classes of nitroreductase have been identified which differ with respect to their sensitivity to oxygen: oxygen-insensitive (Type I) nitroreductases and oxygen-sensitive (Type II) nitroreductases (Djuric *et al.*, 1986; Peterson *et al.*, 1979). The oxygen-insensitive nitroreductases, are comprised of one major component (type IA) and 2 minor components (Types IB₁ and IB₂) (Bryant *et al.*, 1981).

The major type IA nitroreductase referred to as NfsA, (also known as *mdaA*) is encoded by the *nfsA* gene (1.1 kb) located at 19.2 min. on the *E. coli* map (Zenno *et al.*, 1996a). The crystallization and preliminary X-ray crystallographic analysis of NfsA has recently been reported by Kobori *et al.* (1999). NfsA is a flavin mononucleotide (FMN)-containing protein that has a monomeric molecular mass of 26.8 kDa and shares considerable homology with Frp, a flavin oxidoreductase from *Vibrio harveyi* (Zenno *et al.*, 1996a). In terms of nitroreduction, NfsA catalyzes the divalent reduction of nitrosubstituted compounds much more efficiently than NfsB (Yamada *et al.*, 1997). It has recently been reported that NfsA is a member of the *soxRS* regulon and may play a protective role against oxidative stress (Liochev *et al.*, 1999). In addition, it has been shown that NfsA can reduce non-nitro compounds such as quinones, ferricyanide, and 2,6-dichloroindophenol (Liochev *et al.*, 1999).

Recent work from our laboratory has identified and characterized the *nfsA* counterpart in *S. typhimurium* strain TA1535 (Bouramandi *et al.*, in preparation; genbank accession number AF117952). The protein named SnrA, is 27 kDa and shares 93%

identity with *E. coli* NfsA (Bouramandi *et al.*, in preparation). Interestingly, it was previously assumed that all Ames tester strains were isogenic since they were derived from the same parental strain. However, it has been suggested by Yamada *et al.* (1997) that *S. typhimurium* strain TA1538 is missing a key nitroreductase gene. To address this question further, members of the Lambert lab have probed genomic DNA isolated from TA1538 with a radiolabeled *snrA* probe; their results indicate that the *snrA* gene present in TA1535 has been deleted in TA1538 (Bouramandi *et al.*, in prep). The Type II or oxygen-sensitive nitroreductase(s) are believed to play a role in activating certain nitro compounds; however, the genes responsible remain unidentified.

The minor nitroreductase Type IB₁ currently referred to as NfsB, is a FMN-containing flavoprotein with a monomeric molecular mass of 24 kDa and requires either NADH or NADPH as a cofactor (Bryant *et al.*, 1981). The *nfsB* gene is 850 bp and is situated at 13 min. on the *E. coli* map (Zenno *et al.*, 1996a). At the amino acid level, NfsB encoded by the *nfsB* gene of *E. coli* K-12 is 88.5% identical to the 'classical' nitroreductase gene (*cnr*) from *S. typhimurium*. Other homologs have been isolated from *E. coli*, *Enterobacter cloacae*, and are similar to the protein sequence of FRaseI, the major flavin oxidoreductase of *Vibrio fishcheri* (Michael *et al.*, 1994; Yamada *et al.*, 1997; Zenno *et al.*, 1996b). The second minor isoenzyme, type IB₂ has not yet been characterized.

1.3.2 *O*-Acetyltransferase (OAT)

O-acetylation is an important step in the metabolic activation of aromatic amines and nitroarenes (Watanabe *et al.*, 1992). Acetyl CoA-dependent *O*-acetyltransferase/*N*-

acetyltransferase (OAT/NAT) converts *N*-hydroxylamine intermediates into *N*-acetoxyesters, the proximate mutagens. The enzyme is widespread, ranging from bacteria to mammals, including humans (Watanabe *et al.*, 1992). Interestingly, not all bacteria possess an endogenous OAT/NAT activity. Dupret and Grant (1991) found no detectable NAT activity in *E. coli* K-12 derivatives. In humans, there are several known *N*- and *O*-acetyltransferases present in the liver that are involved in the metabolism of drugs or compounds that have an amine or hydrazine group (Watanabe *et al.*, 1992).

In *S. typhimurium*, the *OAT* gene encodes a protein with a molecular mass of 32 kDa and the crystal structure of this protein was recently solved (Watanabe *et al.*, 1992; Sinclair *et al.*, 1998). It is believed that the highly conserved Cys⁶⁹ residue of OAT, and Cys⁶⁸ of human NAT1 and NAT2, are directly involved in the esterification of arylhydroxylamines by acting as an acetyl coenzyme A binding site (Watanabe *et al.*, 1992; Watanabe *et al.*, 1994). Moreover, when Cys⁶⁹ was replaced with alanine, the mutant *S. typhimurium* strain displayed no detectable *O*- and *N*-acetyltransferase activity (Watanabe *et al.*, 1992). The importance of this enzyme in activating nitroarenes and aromatic amines is clearly illustrated by the insensitivity of *S. typhimurium* strain TA98/1,8-DNP₆, an OAT deficient strain, toward these compounds (McCoy *et al.*, 1981; McCoy *et al.*, 1983). Moreover, certain compounds such as the DNPs, absolutely depend on *O*-acetylation for their biological activity. For example a study by McCoy *et al.* (1981) reported 255,790 revertants/nanomole for 1,8-DNP in TA98, while TA98/1,8-DNP₆ yielded only 5,890 reverants/nanomole.

1.3.3 pKM101 and MucA/B

Plasmid pKM101 is a 35 kb N-group plasmid that is present in most of the conventional and new *S. typhimurium* tester strains (Maron and Ames, 1983). The introduction of pKM101 into the Ames tester strains in 1975 greatly increased the sensitivity of the assay (McCann *et al.*, 1975). The plasmid was originally derived from the clinically isolated plasmid R46 and encodes a 1.7 kb region known as *muc* (mutagenesis, UV and chemical) (Perry and Walker, 1982). The *muc* operon encodes two gene products, MucA 16 kDa, and MucB 45 kDa, which are believed to be functional homologs of the chromosomally encoded replicationary accessory proteins, UmuD and UmuC (Perry and Walker, 1982). In *E. coli*, both *mucA/B* and *umuD/C* are closely regulated and are induced as part of the SOS regulatory network. The SOS regulatory network consists of 20 or more unlinked genes e.g. *umuD/C*, *dinA* (*polB*), *uvrA*, *uvrB*, and *uvrD*, that are regulated by the LexA repressor (Walker, 1984). RecA is converted to a coprotease RecA* by an inducing signal such as DNA damage, or a stalled DNA polymerase. RecA* promotes cleavage of the LexA repressor protein, which results in the upregulation of other SOS regulated proteins (Smith and Walker, 1998). Moreover, RecA* mediates the posttranslational processing of UmuD to its mutagenically active form, UmuD' (Smith and Walker, 1998; Shiba *et al.*, 1990). Once activated, UmuD' forms a complex with UmuC and it was postulated that this complex participates in SOS mutagenesis by altering DNA polymerase in such a way that it can bypass DNA lesions either by (1) suppression of DNA polymerase's proofreading functions, (2) an increase in its processivity, or (3) a less stringent requirement for Watson-Crick base pairing (Smith and Walker, 1998). Very recently UmuD'C has been shown to exhibit polymerase

activity *in vitro*. UmuD'C has now been designated as DNA polymerase V (Pol V) (Tang *et al.*, 1999).

At the nucleotide level, the *mucA/B* operon and *umuD/C* operon share 52% identity (Perry *et al.*, 1985). Similarly, in *S. typhimurium* LT2 there are two *umuD/C*-like operons, *S. typhimurium umuD/C* (*umuD/C*_{ST}) and *samAB* (Nohmi *et al.*, 1991). The *samAB* operon is located in a 60-MDa cryptic plasmid that encodes SamA and SamB, functional homologs of UmuDC proteins (Nohmi *et al.*, 1991). At the protein level, SamA and UmuD proteins are 50% similar whereas SamB and UmuC are 62% similar (Nohmi *et al.*, 1991). Mutational specificity studies involving chemical mutagens indicate that the *samA/B* operon does not affect frameshift mutagenesis and is much less efficient at promoting base substitutions than *mucA/B* and *umuD/C* (Watanabe *et al.*, 1994a; Watanabe *et al.*, 1994b).

It has been demonstrated in several mutagenic studies that the presence of pKM101 increases the frequency of base substitutions in both *E. coli* and *S. typhimurium* (Lambert *et al.*, 1992; Rosenkranz and Mermelstein, 1983). Moreover, it appears with aflatoxin B1, 1,8-DNP, and 1,6-DNP that exogenous MucA/B proteins are more efficient at enhancing base substitutions than endogenous UmuD/C and UmuD/C-like proteins (Lambert *et al.*, 1992; Shiba *et al.*, 1990; Urios *et al.*, 1993). Similarly, some mutagens such as aflatoxin B1 are mildly mutagenic in the absence of pKM101, yet are potent carcinogens in the presence of this plasmid (Foster *et al.*, 1988). Not only does pKM101 increase the frequency of base substitutions for some mutagens, it has been recently shown to enhance -2(GC) frameshift events induced by *N*-2-acetylaminofluorene (Janel-Bintz *et al.*, 1994) and -1(A)/+1(A) frameshifts in runs of adenine and/or thymine

(Watanabe *et al.*, 1994a). The enhanced mutagenesis in strains harbouring pKM101 may be explained by work done by Hauser *et al.* (1992) who demonstrated that MucA is posttranslationally processed via RecA* both *in vivo* and *in vitro* much more efficiently than its UmuD homolog. This work contradicts the proposed hypothesis by Shiba *et al.* (1990), who suggested that MucA does not need to be processed to be active in mutagenesis. To date, it has not been shown that MucA'B, like UmuD'C, exhibits DNA polymerase activity.

In a bacterial mutagenicity assay such as the Ames test, it is essential that the strains have functional UmuD/C and/or MucA/B proteins. The mere presence of these proteins increases the number of mutations creating a more sensitive assay. Furthermore, the increased mutagenesis also increases the strains ability to survive the effects of damaged DNA.

1.4 Concerted effects of OAT, nitroreductase, and MucA/B on Ames testing

Major strides have been made in mutation research since the first Ames test was performed in the early 1970s. Through advances in biotechnology, it is now possible to engineer bacterial strains that are more efficient at detecting potential mutagens/carcinogens in complex mixtures. The majority of the work in this area has focused on increasing the sensitivity of the standard Ames tester strains TA98 and TA100 toward nitrosubstituted environmental pollutants. A technique called bioassay-directed fractionation (BDF) combines the use of STTs such as the Ames test, with analytical chemistry techniques. BDF has achieved great success in detecting genotoxins from extracts of urban air particulate, bottom sediments, suspended sediments, and freshwater

mussel tissues (Legzdins *et al.*, 1995). In addition, the Ames test alone has been used extensively in Europe to test industrial wastes and drinking water (reviewed by Houk, 1992; Cerna *et al.*, 1998).

To date, all of the modifications made on the original Ames strains have been undertaken in Takehiko Nohmi's lab in Tokyo, Japan. The first new *S. typhimurium* tester strains YG1021 and YG1026, were constructed by introducing a plasmid (pYG216) carrying the *S. typhimurium cnr* gene (a minor nitroreductase) into both TA98 and TA100 respectively (Watanabe *et al.* 1989). The new strains overproduced nitroreductase and are much more sensitive to certain nitrosubstituted compounds than the previously established strains. In fact, YG1021 (TA98 derivative) was about 70 times more sensitive than YG1020 (TA98 pBR322Ap^r) toward 1-NP (Watanabe *et al.*, 1989).

Subsequently Watanabe *et al.* (1990) constructed two more TA98 and TA100 substrains, YG1024 and YG1029 respectively, which overproduce *O*-acetyltransferase. The increase in OAT activity dramatically elevated the mutagenicity toward some nitrosubstituted compounds. For instance, a 30-fold increase was observed for YG1024 following an Ames test with 1,8-DNP (Watanabe *et al.*, 1990).

The next major advancement was to construct new *S. typhimurium* tester strains that overproduce both the *cnr* nitroreductase and *O*-acetyltransferase, since both enzymes are involved in the metabolism of nitrosubstituted compounds. The new derivatives of TA98 and TA100 i.e. YG1041 and YG1042 were constructed by Hagiwara *et al.* (1993). A 356-fold increase in mutagenicity was observed for YG1041 following an Ames test with 1-NP (Hagiwara *et al.*, 1993). These new strains are currently the most efficient strains available for the detection of nitrosubstituted compounds in the environment.

1.5 Objectives

1.5.1 The first objective of this study was to design and construct several unique plasmids which contain the major and/or minor *E. coli* nitroreductase genes in different combinations with *S. typhimurium* *OAT* and *E. coli* *mucA/B*. These plasmids would contain all the necessary genes for the metabolic activation of nitrosubstituted compounds as well as *mucA/B*. Upon introduction of these plasmids into TA1538 and TA1535, several new sensitive tester strains with a broader substrate specificity would be created.

1.5.2. The second objective of this study was to examine the efficiency of the new strains toward a broad range of nitrosubstituted compounds using the standard Ames test.

2.0 MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and oligonucleotides

The bacterial strains used in this study are listed in Tables (2-5). The plasmids used in this study are listed in Tables (6 and 7). The oligonucleotides used in this study are listed in Table 8.

Table 2. List of *Escherichia coli* strains used in this study

Strain	Characteristics	Source
AB1157	<i>F', thr-1, ura-14, leuB6, lacY1, supE44, galK2, hisG4, tsx-33, rac-, λ, rfbD1, mgl-51, rpsL31, kdgK51, xyl-5, mtl-1, argE3, thi-1, Δ(gpt-proA)62</i>	Bachmann, (1987)
DH5 _α	<i>F'/endA1, hsdR17, (r_k⁻m_k⁺), supE44, thi-1, recA1, gyrA, (Nal^r), relA1 Δ(lacZYA-argF)U169, deoR, (φ80dlacΔ(lacZ)M15)</i>	Woodcock <i>et al.</i> (1989)
JVQ3	same as AB1157, but deficient in <i>nfsA⁻, nfsB⁻</i>	Whiteway <i>et al.</i> , (1998)
JM103	<i>F', traD36, lacI^q, Δ(lacZ)M15, proA⁺B⁺/endA1, supE, sbcBC, thi-1, rpsL, (Str^r) Δ(lac-pro) (P1), (r_k⁻m_k⁺r_{PI}⁻m_{PI}⁻)</i>	Yanisch-Perron <i>et al.</i> (1985)
JM103 Δ <i>umuC</i>	same as JM103 except Δ <i>umuC</i>	Napolitano <i>et al.</i> , (1997)

Table 3. List of established *Salmonella typhimurium* strains used in this study

Strain	Description	Source/Reference
TA1535	<i>hisG46, gal, Δ(chl, uvrB, bio) rfa</i>	B. N. Ames; Ames <i>et al.</i> (1973)
TA1538	<i>hisD3052, gal, Δ(chl, uvrB, bio) rfa</i>	B. N. Ames; Ames <i>et al.</i> (1973)
TA100	TA1535(pKM101)	B. N. Ames; Ames <i>et al.</i> (1973)
TA98	TA1538(pKM101)	D. Bryant; Ames <i>et al.</i> (1973)
YG1021	TA98(pYG216): a Cnr overproducing strain	D. Bryant; Watanabe <i>et al.</i> (1989)
YG1024	TA98(pYG219): an OAT overproducing strain	D. Bryant; Watanabe <i>et al.</i> (1990)
YG1026	TA100(pYG216): a Cnr overproducing strain	D. Bryant; Watanabe <i>et al.</i> (1989)
YG1029	TA100(pYG219): an OAT overproducing strain	D. Bryant; Watanabe <i>et al.</i> (1990)
YG1041	TA98(pYG233): a Cnr and OAT overproducing strain	T. Nohmi; Hagiwara <i>et al.</i> (1993)
YG1042	TA100(pYG233): a Cnr and OAT overproducing strain	T. Nohmi; Hagiwara <i>et al.</i> (1993)

TA strains are derived from *Salmonella typhimurium* LT2 (Maron and Ames, 1983).

Table 4. List of new *S. typhimurium* frameshift tester strains

Strain	Description	Plasmid Encoded Genes	Antibiotic Marker
CFT500	TA1538(pBR322)	none	Tet/Amp
CFT501	TA1538(pGW1700)	<i>mucA/B</i>	Tet
CFT502	TA1538(pBR322A)	<i>nfsA</i>	Tet
CFT503	TA1538(pBR322B)	<i>nfsB</i>	Tet
CFT504	TA1538(pYG219)	<i>OAT</i>	Tet
CFT505	TA1538(pCCOA)	<i>OAT, nfsA</i>	Tet
CFT506	TA1538(pCCOB)	<i>OAT, nfsB</i>	Tet
CFT507	TA1538(pCCOAB)	<i>OAT, nfsA, nfsB</i>	Tet
CFT508	TA1538(pCCOM)	<i>OAT, mucA/B</i>	Tet
CFT509	TA1538(pCCOMA)	<i>OAT, mucA/B, nfsA</i>	Tet
CFT510	TA1538(pCCOMB)	<i>OAT, mucA/B, nfsB</i>	Tet
CFT511	TA1538(pCCOMBA)	<i>OAT, mucA/B, nfsB, nfsA</i>	Tet
CFA504	TA1538(pYG213)	<i>OAT</i>	Amp
CFA505	TA1538(p213A)	<i>OAT, nfsA</i>	Amp
CFA506	TA1538(p213B)	<i>OAT, nfsB</i>	Amp
CFA507	TA1538(p213AB)	<i>OAT, nfsA, nfsB</i>	Amp
CFA508	TA1538(p213M)	<i>OAT, mucA/B</i>	Amp
CFA509	TA1538(p213MA)	<i>OAT, mucA/B, nfsA</i>	Amp
CFA510	TA1538(p213MB)	<i>OAT, mucA/B, nfsB</i>	Amp
CFA511	TA1538(p213MBA)	<i>OAT, mucA/B, nfsA, nfsB</i>	Amp

Table 5. List of new *S. typhimurium* base substitution tester strains

Strain	Description	Plasmid Encoded Genes	Antibiotic Marker
CBT400	TA1535(pBR322)	none	Tet/Amp
CBT401	TA1535(pGW1700)	<i>mucA/B</i>	Tet
CBT402	TA1535(pBR322A)	<i>nfsA</i>	Tet
CBT403	TA1535(pBR322B)	<i>nfsB</i>	Tet
CBT404	TA1535(pYG219)	<i>OAT</i>	Tet
CBT405	TA1535(pCCOA)	<i>OAT, nfsA</i>	Tet
CBT406	TA1535(pCCOB)	<i>OAT, nfsB</i>	Tet
CBT407	TA1535(pCCOAB)	<i>OAT, nfsA, nfsB</i>	Tet
CBT408	TA1535(pCCOM)	<i>OAT, mucA/B</i>	Tet
CBT409	TA1535(pCCOMA)	<i>OAT, mucA/B, nfsA</i>	Tet
CBT410	TA1535(pCCOMB)	<i>OAT, mucA/B, nfsB</i>	Tet
CBT411	TA1535(pCCOMBA)	<i>OAT, mucA/B, nfsB, nfsA</i>	Tet
CBA404	TA1535(pYG213)	<i>OAT</i>	Amp
CBA405	TA1535(p213A)	<i>OAT, nfsA</i>	Amp
CBA406	TA1535(p213B)	<i>OAT, nfsB</i>	Amp
CBA407	TA1535(p213AB)	<i>OAT, nfsA, nfsB</i>	Amp
CBA408	TA1535(p213M)	<i>OAT, mucA/B</i>	Amp
CBA409	TA1535(p213MA)	<i>OAT, mucA/B, nfsA</i>	Amp
CBA410	TA1535(p213MB)	<i>OAT, mucA/B, nfsB</i>	Amp
CBA411	TA1535(p213MBA)	<i>OAT, mucA/B, nfsB, nfsA</i>	Amp

Table 6. List of plasmids used, but not constructed in this study

Plasmid	Size (kb)	Marker	Cloned Genes	Source
pGW1700	6.2	Tet	<i>mucA/B</i>	Perry and Walker, (1982)
pBR322	4.3	Tet/Amp	None	Bolivar <i>et al.</i> (1977)
pYG219	5.7	Tet	<i>OAT</i>	Watanabe <i>et al.</i> , (1990)
pYG213	5.5	Amp	<i>OAT</i>	Watanabe <i>et al.</i> (1990)
pYG216	11.2	Tet	<i>cnr</i>	Watanabe <i>et al.</i> , (1989)
pYG233	10.5	Tet	<i>OAT, cnr</i>	Hagiwara <i>et al.</i> , (1993); Oda <i>et al.</i> , (1993)
pJAB	3.9	Amp	<i>nfsB</i>	Whiteway <i>et al.</i> , (1998)
pATnfsA	5.1	Amp	<i>nfsA</i>	P. Koziaz

Table 7. List of plasmids constructed in this study

Plasmid	Size (kb)	Marker	Cloned Genes
pBR322Aps	3.9	Tet	None
pBR322A	5.2	Tet, Ap ^r	<i>nfsA</i>
pBR322B	4.9	Tet, Ap ^r	<i>nfsB</i>
pCCOB	6.6	Tet	<i>OAT, nfsB</i>
pCCOA	6.9	Tet	<i>OAT, nfsA</i>
pCCOAB	7.7	Tet	<i>OAT, nfsA, nfsB</i>
pCCOM	8.2	Tet	<i>OAT, mucA/B</i>
pCCOMA	9.4	Tet	<i>OAT, nfsA, mucA/B</i>
pCCOMB	9.1	Tet	<i>OAT, nfsB, mucA/B</i>
pCCOMBA	10.2	Tet	<i>OAT, nfsA, nfsB, mucA/B</i>
p213A	6.7	Amp	<i>OAT, nfsA</i>
p213B	6.4	Amp	<i>OAT, nfsB</i>
p213AB	7.5	Amp	<i>OAT, nfsA, nfsB</i>
p213M	8.0	Amp	<i>OAT, mucA/B</i>
p213MA	9.2	Amp	<i>OAT, mucA/B, nfsA</i>
p213MB	8.9	Amp	<i>OAT, mucA/B, nfsB</i>
p213MBA	10.0	Amp	<i>OAT, mucA/B, nfsA, nfsB</i>

Table 8. List of oligonucleotides used in this study

Name	Size (bp)	Sequence 5' - 3'	Description
RmucAB	29	TGCCATCGGATCCATGCTTTTCTGTGACT	Reverse primer for the amplification of <i>mucAB</i>
FmucAB	33	AGAGTTGGATCCCCGAAGAACGTTTCCAAT GA	Forward primer for the amplification of <i>mucAB</i>
MucAB- P	14	ACTGCGCGAGGAAG	<i>mucAB</i> probe complementary to a sequence within the <i>mucB</i> gene

2.2 Chemicals, media, enzymes, and kits

Phenol, boric acid, and tris base, were purchased from Gibco/BRL Life Technologies. Chloroform was purchased from Caledon and isoamyl alcohol was purchased from J. T. Baker Chemical Co. Sodium chloride, sodium acetate, sodium citrate, sodium phosphate, sodium dodecyl sulfate, sodium ammonium phosphate, potassium acetate, potassium phosphate dibasic anhydrous, agar, yeast extract, tryptone, tris-HCl, glycerol, ethylene diamine tetra-acetate, hydrochloric acid, D-glucose, L-histidine, citric acid, magnesium sulfate, were all purchased from BDH Inc. Bovine serum albumin, polyvinylpyrrolidone, dimethylsulfoxide, NADPH, xylene cyanol, bromophenol blue, Ficoll™, D-biotin, Sephadex G-50, were all purchased from Sigma-Aldrich Co. Alcohols were purchased from Commercial Alcohols Inc.

Luria-Bertani (LB) broth (2% w/v LB broth base) (Gibco/BRL Life Technologies Ltd) and LB agar plates 1.5% w/v agar was used to culture all *E. coli* and *S. typhimurium* strains for DNA manipulation. Oxoid nutrient broth #2 (2.5% w/v) (Oxoid Inc.) was used to propagate all overnight *S. typhimurium* cultures for the mutagenicity assay. LB top agar consisted of LB broth base (2% w/v) and agar (0.75% w/v). LB and ONB #2 media were supplemented when required with filter sterilized antibiotics of the following concentrations: ampicillin (100 µg/ml), tetracycline (10 µg/ml), rifampicin (50 µg/ml) and kanamycin (25 µg/ml). All antibiotics were prepared in 95% EtOH and stored at 4 °C, except for rifampicin which was prepared in methanol, and ampicillin which was prepared in dH₂O. All antibiotics were purchased from Sigma-Aldrich Co.

Stock solutions of 50 x VB salts (Vogel and Bonner, 1956), and 40% w/v glucose were prepared and incorporated into minimal glucose media as required. Stock solutions

of 26 mM L-histidine, 0.5 mM biotin, and 0.5 mM histidine/biotin were filter sterilized and stored at 4 °C. Minimal glucose bottom agar consisted of agar (1.5% w/v), 1 x VB salts, and 2.0% glucose while the top agar overlay consisted of agar (0.6% w/v), NaCl (0.5% w/v), and 0.5 mM histidine/biotin. Master plates for the mutagenicity assay consisted of agar (1.5% w/v), 1 x VB salts, 2.0% glucose, 260 µM sterile histidine, 3 µM sterile biotin, and supplemented when required with tetracycline (6.25 µg/ml), ampicillin (50 µg/ml), and kanamycin (50 µg/ml).

All test chemicals used in this study were prepared fresh as required in DMSO. The test chemicals were: 4-nitroquinoline-1-oxide, ethyl methanesulfonate, ICR-191 (2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine·2HCl), nitrofurazone, 1-nitropyrene, 1,6-dinitropyrene, 2-nitronaphthalene, and 2-nitrofluorene. All were purchased from Sigma-Aldrich Co. except for 1,6-DNP which was a generous gift from Douglas Bryant.

All restriction enzymes and DNA modification enzymes were purchased from either New England Biolabs or Gibco/BRL Life Technologies Ltd. Expand™ DNA polymerase, related buffers, dNTPs, RNase, agarose, and glycogen were purchased from Roche Diagnostics. Oligonucleotides for PCR and probing (see Oligos, Table 7) were synthesized by Gibco/BRL Life Technologies Ltd. and BIO/CAN Scientific.

Plasmid DNA was extracted initially using a Circle prep kit (Bio101), but later the Wizard® plus miniprep DNA purification system (Promega) and a vacuum manifold was adopted.

2.3 Sources of *nfsB*, *nfsA*, and *mucA/B*

All cloning experiments were performed in either DH5 α or JM103 Δ *umuC*. Restriction endonucleases and DNA modification enzymes were supplied by either New England Biolabs or Gibco/BRL Life Technologies. The enzyme reactions were carried out under the conditions recommended by the manufacturer.

2.3.1 *nfsB*

The original source of *nfsB* was *E. coli* strain AB1157. Jacqui Whiteway isolated and cloned *nfsB* (850 bp) into the *EcoR* I and *Bam*H I site of pUC118, the resulting plasmid was named pJAB. pJAB was digested with *EcoR* I and *Acc* I to release an 860 bp fragment which contained *nfsB*. The digest was electrophoresed (BioRad minicell EC370M) on a 0.7% agarose gel containing EtBr (0.3 μ g/ml) in 1 x TAE buffer (0.04 M tris-acetate, 0.001 M EDTA, pH 8.0) at 79 V for 45 minutes. The band was visualized on a Fisher Biotech 312 nm transilluminator, excised with a razor blade and purified using the Gene Clean[®] (Bio 101) procedure. DNA containing the *nfsB* gene was precipitated overnight in 1 ml of cold 0.16 M EtOHNaC supplemented with 6 μ l of glycogen (2 μ g/ μ l) (Roche Diagnostics) at -20 °C. The purified *nfsB* DNA served as the source of *nfsB* for all subsequent cloning experiments.

2.3.2 *nfsA*

The original source of *nfsA* was *E. coli* strain AB1157. Peter Koziarz isolated and cloned *nfsA* (1165 bp) into the TA cloning vector pCR[®]2.1 (Invitrogen) which was re-named pATnfsA. An *EcoR* I digest released *nfsA* from pATnfsA and the *nfsA* digest was

electrophoresed on a 0.7% agarose gel containing EtBr (0.3 µg/ml) and the *nfsA* fragment was excised and purified using the Gene Clean procedure. Pure *nfsA* DNA was precipitated overnight in 1 ml of cold 0.16 M EtOH₄Nac supplemented with 6 µl of glycogen (2 µg/µl) at -20 °C. The purified *nfsA* DNA then served as the source of *nfsA* for all subsequent cloning experiments.

2.3.3 *mucA/B*

The source of *mucA/B* was pGW1700 which was kindly provided by Dr. G. C. Walker. The *mucA/B* gene was amplified using PCR with primers RMucAB and FMucAB (see Oligo Table 8) and the Expand™ High Fidelity PCR System (Roche Diagnostics). The PCR amplification was carried out using an Amplitron® II Thermolyne PCR machine (Barnstead). The PCR reactions were done in 50 µl volumes using the following conditions: 1x Expand™ buffer, 10 mM dNTPs, 25 mM MgCl₂, 15 pmol of each primer, 0.75 units of Expand™ DNA polymerase, and 0.04 µg DNA template. A negative control was also prepared replacing pGW1700 DNA with ddH₂O. The PCR was performed according to the following program: predwell at 94 °C for 5 minutes, followed by 20 cycles of 94 °C for 5 minutes; 55 °C for 30 seconds; 72 °C for 1 minute. A postdwell at 72 °C for 5 minutes was performed on all samples and the samples were maintained at 4 °C until use.

The PCR product (~ 2.5 kb) containing the *mucA/B* gene (1.8 kb) was visualized on a 0.7% agarose gel containing EtBr and purified using a High Pure PCR purification kit (Roche Diagnostics) according to the manufacturer's protocol. This became the source of *mucA/B* for subsequent cloning experiments.

2.3.4 Klenow treatment of *mucA/B*

DNA containing *mucA/B* was treated with DNA polymerase I large (Klenow) fragment (New England Biolabs) to fill-in 3' recessed ends for further cloning. The fill-in reaction consisted of the following: 1 x Klenow buffer, 1.0 mM dNTPs, 5 units of DNA polymerase I large fragment (Klenow), and 5 µg of pure *mucA/B*. The reaction was incubated at 25 °C for 25 minutes. The reaction was stopped by a phenol/chloroform (25:25:1) extraction, 6 µl of glycogen (2 µg/µl) was added and the DNA was precipitated overnight at -20 °C using 0.16 M ETOHNaC.

2.4 Plasmid construction

All the constructs created in this work (except pBR322Aps, pBR322A, and pBR322B) were derived from two plasmids, pYG219 and pYG213 (Watanabe *et al.*, 1990) (see plasmid Figs. 6 and 7); kindly provided by Dr. P. D. Josephy. Both plasmids are derivatives of pBR322 and contain the *OAT* gene. In pYG213, *OAT* was cloned into the *EcoR* V/*Bam*H I sites within the tetracycline gene, thus inactivating this gene, while in pYG219 *OAT* was cloned into the *Sca* I site situated within the ampicillin gene.

All gene cloning experiments consisted of a 10 µl ligation reaction containing 1 pmol vector to 3 pmol insert and 400 U of T4 DNA ligase (New England Biolabs). Each reaction was incubated overnight at 13-16 °C and 1-2 µl of this reaction was used to transform 40 µl of electro-competent DH5 α cells in 0.2 cm electroporation cuvettes (Invitrogen); for some *mucA/B* ligations electro-competent JM103 Δ *umuC* cells were used. Transformations were done using a BioRad *E. coli* Pulser set at 2.5 kV. Immediately after electroporation, 960 µl of SOC medium (2% tryptone, 0.5% yeast

extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added to the cells and they were immediately placed in a 37 °C shaker bath set at 200 RPM for at least 20 minutes. The cells were then diluted and plated onto LB plates supplemented with either ampicillin (100 µg/ml) or tetracycline (10 µg/ml) and incubated at 37 °C for 24 hours. Transformants were randomly selected and grown overnight in LB broth supplemented with the appropriate antibiotic. Plasmid DNA was extracted using the Wizard® plus miniprep DNA purification system (Promega) and a vacuum manifold. The DNA was then electrophoresed on a 0.7% agarose gel containing EtBr (0.3 µg/ml) along with a supercoiled DNA ladder (Gibco BRL). Cells harbouring plasmids of the expected size were streaked onto plates for further analysis. Plasmid size was the primary screening method used.

The functionality of cloned nitroreductase genes was determined by a nitrofurazone filter disc assay (see section 2.9) while cloned *mucA/B* genes were determined using a UV rifampicin assay (see section 2.7).

2.5 Construction of plasmids conferring tetracycline resistance

2.5.1 Construction of pCCOB

Plasmid pCCOB was created by inserting the 860 bp *EcoR* I/*Acc* I *nfsB* fragment (see section 2.3.1) from pJAB into the *EcoR* I/*Cla* I sites of pYG219. *Acc* I and *Cla* I ends are compatible. The expected plasmid size of pCCOB was 6.6 kb.

2.5.2 Construction of pCCOA

Plasmid pCCOA was constructed by inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) from pATnfsA into the *EcoR* I site of pYG219. The expected size of pCCOA was 6.9 kb. The orientation of *nfsA* was confirmed by a *Kpn* I and *Cla* I restriction digest.

2.5.3 Construction of pCCOAB

Plasmid pCCOAB was constructed by digesting pCCOB to completion with *EcoR* I and inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) into this site. The expected size of pCCOAB was 7.7 kb. The orientation of *nfsA* was confirmed by a *Kpn* I restriction digest.

2.5.4 Construction of pCCOM

Plasmid pCCOM was constructed by inserting a Klenow-treated *mucA/B* PCR fragment (see section 2.3.3 and 2.3.4) into the *Msc* I site of pYG219. The expected size was 8.2 kb. The orientation of *mucA/B* was determined by an *Ava* I restriction digest.

2.5.5 Construction of pCCOMB

Plasmid pCCOMB was constructed by inserting a Klenow-treated *mucA/B* PCR fragment (see section 2.3.3 and 2.3.4) into the *Msc* I site of pCCOB. The expected size was 9.1 kb. The orientation of *mucA/B* was determined by an *Ava* I restriction digest.

2.5.6 Construction of pCCOMA

Plasmid pCCOMA was constructed by inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) into the *EcoR* I site of pCCOM. The expected plasmid size was 9.4 kb. The orientation of *nfsA* was determined by a *Kpn* I and *Nhe* I restriction digest.

2.5.7 Construction of pCCOMBA

Plasmid pCCOMBA was constructed by inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) into the *EcoR* I site of pCCOMB. The expected size was 10.2 kb. The orientation of *nfsA* was determined by a *Kpn* I restriction digest.

2.5.8 Construction of pBR322Aps

Plasmid pBR322Aps was constructed by digesting pBR322 (Bolivar *et al.*, 1977) to completion with *Sca* I and *Ssp* I to remove a 400 bp fragment within the ampicillin gene. The expected size was 3.9 kb.

2.5.9 Construction of pBR322A

Plasmid pBR322A was constructed by inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) into the *EcoR* I site of pBR322Ap^r. The expected plasmid size was 5.2 kb. The orientation of *nfsA* was determined by a *Kpn* I and *Cla* I restriction digest.

2.5.10 Construction of pBR322B

Plasmid pBR322B was constructed by inserting an *EcoR* I/*Acc* I fragment containing *nfsB* (see section 2.3.1) into the *EcoR* I/*Cla* I site of pBR322Ap^r. The expected plasmid size was 4.9 kb.

2.6 Construction of plasmids conferring ampicillin resistance

2.6.1 Construction of p213A

Plasmid p213A was constructed by digesting pYG213 to completion with *EcoR* I and inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) into this site. The expected plasmid size of p213A was 6.7 kb. The orientation of *nfsA* was confirmed by a *Kpn* I and *Cla* I restriction digest.

2.6.2 Construction of p213B

Plasmid p213B was constructed by inserting an *EcoR* I/*Acc* I fragment containing *nfsB* (see section 2.3.1) into the *EcoR* I/*Cla* I sites of pYG213. The expected plasmid size of p213B was 6.4 kb.

2.6.3 Construction of p213AB

Plasmid p213AB was constructed by inserting an *EcoR* I fragment containing *nfsA* (see section 2.3.2) into the *EcoR* I site of p213B. The expected size of p213AB was 7.5 kb. The orientation of p213AB was determined by a *Kpn* I restriction digest.

2.6.4 Construction of p213M

Purified *mucA/B* PCR product (see section 2.3.3) was digested with *Bam*H I and inserted into the *Bam*H I site of pYG213. The expected size of p213M was 8.0 kb. The orientation of *mucA/B* was determined by an *Ava* I restriction digest.

2.6.5 Construction of p213MB

The construction of p213MB was accomplished by treating purified *mucA/B* PCR product (see section 2.3.3) with Klenow fragment (New England Biolabs) (see section 2.3.4) to generate blunt ends and inserting this fragment into the *Msc* I site of p213B. The expected plasmid size was 8.9 kb. The orientation of *mucA/B* was determined by an *Ava* I restriction digest.

2.6.6 Construction of p213MA

Plasmid p213MA was constructed by inserting an *Eco*R I fragment containing *nfsA* (see section 2.3.2) into the *Eco*R I site of p213M. The expected plasmid size was 9.2 kb. The orientation of *nfsA* was determined by a *Kpn* I and *Cla* I restriction digest.

2.6.7 Construction of p213MBA

Plasmid p213MBA was constructed by inserting an *Eco*R I fragment containing *nfsA* (see section 2.3.2) into the *Eco*R I site of p213MB. The expected plasmid size was 10.0 kb. The orientation of *nfsA* was determined by a *Kpn* I restriction digest. The orientation of *mucA/B* was determined by an *Ava* I restriction digest.

2.7 γ -³²P oligonucleotide probing for *mucA/B* clones

2.7.1 Preparation of Sephadex G-50 column

A 3 cc syringe with plunger removed was packed with a small piece of glass wool and the syringe was carefully filled with Sephadex G-50 coarse resin without the introduction of air bubbles. The entire column was placed inside a 13 mm x 100 mm glass tube with a 1.5 ml eppendorf tube at the bottom to collect the eluate. The column was centrifuged at low speed for 5 minutes in a Janetzki T30 clinical centrifuge.

2.7.2 Labelling of *mucA/B* for probing

Oligonucleotides containing 17 base pairs of the *mucA* gene (see Oligo Table 8) were end labeled in a reaction consisting of γ -³²P-ATP in a ratio of 1:20 (hot ATP: cold ATP): 2 μ l *mucA/B*-P (14-mer) (200 pmol), 1 μ l ATP (1 nmol) (1000 pmol), 30 μ l γ -³²P-ATP (50 pmol), 5 μ l 10x PNK buffer, 7 μ l dH₂O, and 5 μ l polynucleotide kinase (50 U) (New England Biolabs). The reaction was incubated in a Hybaid combi thermal cycler TR2 for 60 minutes at 37 °C. The reaction was heat inactivated by incubation at 75 °C for 15 minutes and 50 μ l of TE was then added. Unincorporated ³²P-labelled ATP was removed by passing the reaction through a G-50 column via low speed for 5 minutes and 300 μ l of TE was added to the eluate. The final concentration of *mucA/B*-P was 0.5 pmol/ μ l. The probe was stored at -20 °C until needed.

2.7.3 Preparation of Hybond-N membranes

Transformants from the pYG213:*mucA/B* ligation and or pYG219:*mucA/B* ligation were gridded onto 4 LB plates (195 colonies/plate) along with 2 positive controls for *mucA/B* (pGW1700) and 2 negative controls (DH5 α). The cells were grown for 24 hours at 37 °C. The colonies were lifted after placing Hybond-N nitrocellulose membranes (Amersham) on top of the colonies. The membranes were then transferred using forceps to a 10 cm x 40 cm strip of 3mm chromatography paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and let sit for 3-5 minutes. The membranes were then transferred to another strip of 3 mm chromatography paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M tris-HCl pH 7.2, 0.001 M EDTA) for 5-6 minutes. The membranes were treated again with neutralizing solution and were finally transferred to chromatography paper soaked in 2 x SSC (0.3 M NaCl, 0.03 M Na₃citrate) for 5-6 minutes. The membranes were dried in a 75 °C oven for 5 minutes and then placed face down on a Fisher Biotech 312 nm transilluminator with the UV lamp on for 20-30 minutes. The membranes were folded so that the colonies were facing each other and placed inside a 50 ml Falcon tube containing 25 ml of hybridization solution that contained 6.25 ml of 20 x SSPE (21.1% w/v NaCl, 2.8% w/v sodium phosphate, 0.7% w/v EDTA, pH 7.7), 2.5 ml of 50 x Denhardt's solution (1% (w/v) bovine serum albumin, 1% (w/v) Ficoll™ 400, 1% (w/v) polyvinylpyrrolidone), 10% (w/v) SDS, 15 ml ddH₂O, 50 μ l (50 μ g/ μ l) herring sperm ss DNA and 15 μ l of the radiolabeled probe (0.5 pmol/ μ l). Two Falcon tubes were inserted into a hybridization tube and placed in a hybridization oven (VWR Scientific model 2710) and rotated at low speed for 24 hours at 37 °C.

2.7.4 Washing of membranes

The hot hybridization solution was poured into ^{32}P liquid waste, and the tubes were re-filled with 2 x SSPE, 0.1% SDS and allowed to stand for 10 minutes. The solution was removed and the membranes were washed a second time with 2 x SSPE, 0.1% (w/v) SDS followed by one final wash with 0.1 x SSPE, 0.1% (w/v) SDS pre-warmed to 50 °C. The signal from the membranes was checked with a Biodex #3 Geiger counter and the membranes were prepared for autoradiography.

2.7.5 Preparation of membranes for autoradiography

The membranes were dried by pressing each membrane down on a piece of 3 mm chromatography paper. A glass sequencing plate was covered with plastic wrap and 2 membranes were placed side by side facing down on the plate. Using a razor blade the plastic wrap was cut in half and carefully folded over the 2 membranes and taped. The wrapped membranes were then placed into an X-ray cassette (35 cm x 43 cm) containing an intensifying screen and secured to a used film with tape. All membranes were secured inside the cassette and a sheet of Kodak BioMax MR-1 film (Amersham) was placed on top of them. The entire cassette was placed in a -80 °C freezer for 24-48 hours. The film was processed manually using Kodak fixer and developer (Picker) at room temperature.

2.8 UV rifampicin assay for *mucA/B*

The UV rifampicin assay was used to determine whether cloned *mucA/B* genes were functional. For this test, plasmids containing *mucA/B* were transformed into JM103 $\Delta umuC$ by electroporation. The positive control was JM103 $\Delta umuC$ pGW1700

(*mucA/B*⁺) and the negative control was JM103 Δ *umuC* pYG219 (*mucA/B*⁻). Overnight cultures were grown in 6 ml LB broth tubes supplemented with the appropriate antibiotic (ampicillin 100 μ g/ml; tetracycline 10 μ g/ml) at 37 °C and 200 RPM. The next morning 50 ml of LB broth supplemented with antibiotic was inoculated with 0.5 ml of O/N culture. The cells were grown at 37 °C until the O.D.₆₀₀ was about 0.9, approximately 2-3 hours. The cells were centrifuged in a Sorval® RC-5B refrigerated superspeed centrifuge (Dupont Instruments) for 5 minutes at 10,000 RPM and the supernatant was discarded. The cells were resuspended with 2 ml of cold 10 mM MgSO₄, and centrifuged again. The pellet was resuspended in 2 ml of 10 mM MgSO₄ and a further 23 ml of 10 mM MgSO₄ was added and the entire suspension was poured into a petri dish.

Tubes containing 6 ml of LB broth and supplemented with antibiotic were wrapped with tin foil to prevent light from entering the tubes. In the dark room, a 250 μ l aliquot of cells was taken and added to a labeled, foil-covered, LB tube (0 time). Cells were suspended in a petri dish and exposed to UV light (73 μ W/cm²) (Mineralight UV light model R-52) using an UV light and nutator. The UV energy measurement was taken with a UVX radiometer. Aliquots of 250 μ l were taken every 15 seconds for 2 minutes and placed into the appropriately labeled LB tubes. The tubes were placed in a 37 °C shaker for O/N amplification at 200 RPM. The next morning the cultures were plated onto LB plus antibiotic (to give total number of viable cells) and onto LB rifampicin media (50 μ g/ml) (to give total induced rifampicin mutants). Cells that grow on rifampicin have suffered a mutation, likely to the β subunit of RNA polymerase, preventing rifampicin from binding to the β subunit (Nene and Glass, 1982). Serial dilutions were made for each time point and each dilution was plated in duplicate. The

cells were then counted using a Quebec colony counter (New Brunswick Scientific Co.) or an AccuCount 1000 automatic colony counter (Biologics). The counts were converted into CFU/ml and averaged for each dilution. The ratio of induced rifampicin mutants:viable cells was calculated as follows:

$$\frac{\text{average CFU/ml (rifampicin)}}{\text{average CFU/ml (LB + antibiotic)}}$$

2.9 Transformation of JVQ3 cells with plasmids

JVQ3 cells (*nfsA*⁻, *nfsB*⁻) were transformed with each plasmid using the CaCl₂ method of transformation. An aliquot of pure plasmid DNA, usually 2 µl was mixed with 40 µl of JVQ3 cells that had been previously prepared using 0.1 M MgCl₂, and 0.1 M CaCl₂ and frozen at -20 °C. The cells and DNA were set to stand on ice for 1 hour. The cells were heat shocked for 5 minutes at 37 °C and immediately placed back on ice for 5 minutes. An aliquot of LB broth was added (960 µl) to the cells and they were placed into a 37 °C shaker set at 200 RPM for at least 1 hour. A 100 µl aliquot of cell suspension was diluted and plated onto LB media supplemented with the appropriate antibiotic and placed into a 37 °C incubator for 24 hours.

2.10 Nitrofurazone filter disc assay using JVQ3 cells

A nitrofurazone filter disc assay was used to determine the functionality of cloned nitroreductase genes. Constructs containing cloned *nfs* genes were transformed into JVQ3 cells (*nfsA*⁻ *nfsB*⁻) using the CaCl₂ method. Overnight cultures were grown in LB broth supplemented, when necessary, with ampicillin (100 µg/ml) or tetracycline (10 µg/ml) for plasmid selection and maintenance. LB top agar was prepared and 4 ml was

dispensed into test tubes and stored at 4 °C until use. From an overnight culture, 100 µl was added to 4 ml of molten top agar held at 45 °C, vortexed and quickly poured onto a warm LB plate. Once the top agar hardened, a sterile 3 mm Whatman filter disc (0.6 cm in diameter) was placed onto the surface of the plate using sterile forceps. Ten microliters of nitrofurazone 5.5 µg/µl dissolved in DMSO was added to the disc and the plate was incubated upright at 37 °C for 24 hours. The clearing or killing zone was measured using a standard ruler and the mean values were reported.

2.11 Transformation of plasmids into *Salmonella typhimurium* TA1535 and TA1538

Plasmid DNA (1-2 µl) was transformed by electroporation into 40 µl of electro-competent TA1535 cells. The electroporation was performed in 0.2 cm electroporation cuvette (Invitrogen) and a BioRad *E. coli* Pulser set at 2.5 kV. Immediately after shocking the cells, 960 µl of SOC medium was added to the cells and they were placed in a 37 °C shaker bath set at 200 RPM for at least 20 minutes. The cells were then diluted and plated onto LB plates supplemented with the appropriate antibiotic and incubated at 37 °C for 24 hours. Transformants were randomly selected and grown in overnight cultures of LB broth supplemented with the appropriate antibiotic. Frozen permanent stocks suspended in 15% glycerol in LB were prepared and stored at -80 °C.

Plasmid DNA transformed into TA1538 was first extracted from TA1535 and then transformed into electro-competent TA1538 cells. The direct transformation of plasmid DNA into TA1538 routinely yielded no transformants.

2.12 Nitrofurazone reductase assay

The nitrofurazone reductase assay was used to determine the specific activity of the newly constructed *S. typhimurium* tester strains. Two independent overnight cultures of each *S. typhimurium* tester strains constructed (as described in section 2.8) were grown in 6 ml of ONB #2 (2.5% w/v) supplemented with antibiotic. The cultures were centrifuged in a clinical centrifuge (IEC HN-SII) and the pellets resuspended in 250 µl of 50 mM tris-HCl pH 7.0 and placed on ice prior to sonication. Using a Kontes microultrasonic cell disrupter, the pellets were sonicated 2 times each for 30 second intervals. After sonication, the extracts were then centrifuged in an Eppendorf 5404 centrifuge set at 15, 000 RPM for 30 minutes at 4 °C. The supernatant was removed and added to a new 1.5 ml eppendorf tube and placed on ice until needed or stored at -20 °C.

The protein concentration for all cell free extracts was determined by the method of Lowry *et al.* (1951). A standard curve for BSA (Sigma-Aldrich Corporation) was prepared using an Ultrospec 3000 set at 595 nm (Pharmacia Biotech). The BSA samples were done in triplicate and were comprised of 800 µl of ddH₂O, 200 µl of Bradford reagent (BioRad) and 2 µl of diluted BSA. Tubes were inverted 2 times and incubated for 5 minutes at room temperature. A blank containing only the Bradford reagent and ddH₂O was used to reference the spectrophotometer. Absorbance readings were taken for each sample and the average was used to calculate the amount of protein according to the following equation:

$$\text{Abs @ 595}/(\text{slope of standard curve})/2 = \text{mg/L}.$$

Once the standard curve was completed, the protein concentrations for each cell free extract was determined. The nitrofurazone reductase assay was carried out in

Ultrospec 3000 (Pharmacia Biotech) set at a wavelength of 400 nm. The molar extinction coefficient for nitrofurazone at 400 nm is $12,960 \text{ M}^{-1} \text{ cm}^{-1}$. Each reaction received 0.1 mM NADPH, 0.1 mM nitrofurazone in DMSO, 45 μl tris-HCl, 50 μl of cell free extract, and ddH₂O to a final volume of 1 ml. The reactions were inverted twice and placed immediately into the spectrophotometer. Absorbance readings were taken every 10 seconds for 100 seconds and the slope of the line (change in absorbance over time) was determined by the spectrophotometer (see example in Fig. 18). Some samples required a 10-fold and even a 100-fold dilution in order to achieve a linear line. Each cell free extract was performed twice from two independent inoculations and the specific activity for each was calculated as follows:

$$\begin{aligned} \text{Specific Activity} &= \frac{\text{Result}(\mu\text{mol reduced/min/L})}{[\text{protein concentration (mg/L)}]} \\ &= (\mu\text{mol nitrofurazone reduced/min/mg protein}) \end{aligned}$$

2.13 Mutagenicity Assay (Ames test)

The mutagenicity test was carried out in a Bioklone laminar flow hood. Overnight *S. typhimurium* tester strains were grown with shaking in ONB #2 supplemented with ampicillin (50 $\mu\text{g/ml}$) or tetracycline (6.25 $\mu\text{g/ml}$) as required. A digital timer was connected to the shaker to ensure a growth period of 12 hours. Minimal glucose agar plates were poured by hand or by using an AgarMatic bench-top agar sterilizer (New Brunswick Co. Inc) and a PourMatic model MP-320 plate pourer (New Brunswick Co Inc.) set at 25 ml/plate. Top agar was prepared and transferred to sterile snap cap Falcon tubes in 2 ml portions using a Brinkman multipipettor fitted with a 50 ml combitip. The top agar was melted using a hotblock and the tubes were placed in a 45 °C

water bath until needed. Mutagens were weighed out and dissolved in DMSO the day of the experiment, diluted, and used immediately. An aliquot was taken from each tester strain, diluted, and plated onto LB plates supplemented with appropriate antibiotic to determine the number of viable cells (CFU/ml) (see Fig. 1). To the molten top agar, a 100 μ l aliquot of mutagen and a 100 μ l aliquot of tester strain were added, vortexed, and immediately poured onto warm minimal glucose agar. The plates were placed into a cardboard box, sealed and incubated for 48 hours in the dark at 37 °C. The colonies were counted on an AccuCount 1000 automatic colony counter (Biologics) with correction switch on. Each dose was done in triplicate and each plate was counted three times for a total of 9 trials. The mean number of histidine revertants per plate was recorded by subtracting out the spontaneous or DMSO treated colony counts. A dose response curve was plotted for some strains and the highest number of induced revertants/nanomole taken from the linear portion of the dose response curve was reported for each strain. Positive controls were 4-NQO (MW 190.16) for TA1535 derivatives and 2-NF (MW 211.22), for TA1538 derivatives. The test chemicals were made fresh the day of the experiment in DMSO and they consisted of 1,6-DNP (MW 292.25), 1-NP (247.26), NF (MW 198.1), 2 NN (173.17), ICR-191, (MW 451.2) and EMS (MW 124.2).

3.0 RESULTS

3.1 Construction of plasmids

The plasmids were constructed as described in sections 2.4 and 2.5. Restriction digests were used to determine the orientation of cloned *nfsA* and *mucA/B* genes. Macplasmmap version 1.8 © 1988 was used to design all 20 plasmid maps. The plasmids were divided into 5 groups. Group I was comprised of ampicillin constructs without *mucA/B* (Fig. 8), Group II was comprised of tetracycline constructs without *mucA/B* (Fig. 9), Group III was comprised of ampicillin constructs with *mucA/B* (Fig. 10), Group IV was comprised of tetracycline constructs with *mucA/B* (Fig. 11), and Group V was comprised of pBR322 tetracycline constructs (Fig. 12).

3.2 Nitrofurazone filter disc assay

Plasmids were transformed into *E. coli* JVQ3 cells (*nfsA*⁻, *nfsB*⁻) (as described in section 2.8) to verify that the cloned nitroreductase genes were functional. An aliquot of cells was mixed with molten LB top agar and poured onto LB plates. Several small sterile Whatman filter discs were placed on the hardened top agar and nitrofurazone was applied to each disc (as described in section 2.9). Following an overnight incubation, a clearing zone was evident around each paper disc (Fig. 13). The diameter of the clearing zone was measured and the diameter of the filter disc was subtracted out for each plasmid. The results are shown in Fig. 14. The JVQ3 cells alone, pBR322, and pGW1700 all showed a slight response indicating another endogenous nitroreductase is probably present in the JVQ3 cells. In general, there is a natural progression toward a larger killing zone for several groups of plasmids. In group I, with the exception of

Figure 8. A schematic representation of Group I plasmids. Each of these plasmids was originally derived from pYG213 and encode an ampicillin resistance marker. This group of plasmids express *OAT*, and *nfsA* and/or *nfsB* genes.

Figure 9. A schematic representation of Group II plasmids. Each of these plasmids was originally derived from pYG219 and encode a tetracycline resistance marker. This group of plasmids express *OAT*, and *nfsA* and/or *nfsB* genes.

Figure 10. A schematic representation of Group III plasmids. Each of these plasmids was originally derived from pYG213 and encode an ampicillin resistance marker. This group of plasmids express *mucA/B*, *OAT*, and *nfsA* and/or *nfsB* genes.

Figure 11. A schematic representation of Group IV plasmids. Each of these plasmids was originally derived from pYG219 and encode a tetracycline resistance marker. This group of plasmids express *mucA/B*, *OAT*, and *nfsA* and/or *nfsB* genes.

Figure 12. A schematic representation of Group V plasmids. Each of these plasmids was originally derived from pBR322 and encode a tetracycline resistance marker. This group of plasmids express *mucA/B*, or *nfsA*, or *nfsB*.

Figure 13. A scanned image representing the nitrofurazone filter disc assay. An overlay of LB top agar containing an aliquot of *E. coli* JVQ3 (*nfsA*⁻, *nfsB*⁻) cells plus several transformed plasmids containing different combinations of *OAT* and nitroreductase genes. NF dissolved in DMSO was applied to the disc and the plates were incubated overnight at 37 °C. The killing zones were measured and the diameter of the disc was subtracted. A red background was used to enhance the image during scanning.

- (1) JVQ3 (p213AB)
- (2) JVQ3 (pCCOMBA)
- (3) JVQ3 (pYG219)
- (4) JVQ3 (pCCOAB)
- (5) JVQ3 (pMOAP now denoted as p213MBA)

1

5

2

4

3

Figure 14. A graphic representation of the mutagenic response of *E. coli* JVQ3 (*nfsA*⁻, *nfsB*⁻) cells with NF following the transformation of these cells with plasmids containing different combinations of nitroreductase genes, *OAT*, and *mucA/B*. The red bars represent plasmids encoding an ampicillin resistant marker while the blue bars represent plasmids encoding a tetracycline resistant marker. The error bars represent the standard deviation.

p213B, the addition of nitroreductase genes increased the killing zone. The same trend was observed with Group II, Group III, and Group V plasmids. However, Group IV plasmids did not conform to this trend. Overall Group II and Group IV plasmids showed a larger killing zone than their ampicillin counterparts. Moreover, pCCOAB had the largest killing zone out of all the plasmids tested. The presence of *mucA/B* on a plasmid did not have a consistent effect on toxicity. The presence of *nfsA* increased the killing zone dramatically in all plasmid groups. The *cnr* plasmid pYG216 was included to compare a *Salmonella* minor nitroreductase gene to both the major and minor from *E. coli*. The killing zone from pYG216 was comparable to several *nfsA* containing plasmids, and greater than all plasmids bearing *nfsB*, the *E. coli* *cnr* homolog.

The major nitroreductase gene from *E. coli* enhanced the metabolic capacity of the *S. typhimurium* tester strains substantially when it was introduced into these strains. A statistical comparison using a two-sample unequal variance T-test was made between strains lacking *nfsA* containing plasmids, and strains harbouring *nfsA* containing plasmids. For example, p213 versus p213A (data not shown). The results of this test were always statistically significant with P values ranging between $0.05 < P < 0.001$.

3.3 UV rifampicin assay for *mucA/B* functionality

The UV rifampicin assay was used to determine whether the cloned *mucA/B* genes were functional. The potential clones were identified by autoradiography (Fig. 15) and transformed into *E. coli* JM103 Δ *umuC* so that the effects of *mucA/B* could be clearly observed. Following UV treatment and an overnight growth period the cells were plated

Figure 15. A scanned image representing *mucA/B* probing results for pCCOMB. Several positive clones were picked from the original plate and plasmid DNA was extracted. The correct plasmid size was verified by gel electrophoresis. The orientation of *mucA/B* was determined by restriction digests. The lines on the image were used to orient the membrane with the original plate. The positive control was *E. coli* JM103 Δ *umuC* pGW1700 cells which are represented by the arrow at the top of the image. The negative control was *E. coli* JM103 Δ *umuC* pYG219 and is represented by the arrow at the left side of the image.

and the colonies were counted (as described in section 2.7). Line graphs were plotted to illustrate the effects of cloned *mucA/B* genes (Figs. 16 and 17). Plasmids p213M and p213MB were tested for functional MucA/B proteins; both p213M and p213MB showed a UV dose dependent increase in the frequency of formation of rifampicin resistant mutations indicating the presence of functional MucA/B proteins (Fig. 10). Similarly, as shown in Fig. 17, both pCCOM and pCCOMB displayed a dose dependent increase in UV induced mutation. No increase in UV induced mutation was observed in the negative controls (JM103 $\Delta umuC$ pYG213 and JM103 $\Delta umuC$ pYG219) illustrating the requirement for MucA/B gene products (or the UmuD/C homologs) in UV mutagenesis.

3.4 Nitrofurazone reductase assay

Nitrofurazone reductase was used to measure nitroreductase activity encoded by cloned nitroreductase genes (see section 2.12). The nitrofurazone reductase activity was determined for each new tester strain and the previously established strains. The enzyme assays were performed in duplicate from two independent inoculations and the specific activities were averaged (Tables 9 and 10). Figure 18 represents an example of a printout from the Ultrospec 3000 depicting a typical result following the reduction of nitrofurazone.

In general, the nitrofurazone reductase data was consistent, as evidenced by the low standard deviations. However, certain strains which expressed a high level of nitroreductase activity from either the *cnr* or *nfsA* genes had relatively high standard deviations. Overall, the strains containing Group II and Group IV plasmids (tetracycline

Figure 16. The effects of p213M (green) and p213MB (black) on *E. coli* JM103 Δ *umuC* strains following UV treatment. The cells were treated with UV for a period of 2 minutes and aliquots were taken every 15 seconds. The cells were then grown overnight in the absence of light and plated onto LB and LB + rifampicin. The negative control (red) is deficient in *mucA/B* and the positive control (blue) contains a plasmid encoding *mucA/B*. The number of induced rifampicin mutants are expressed as a ratio of average rifampicin mutants/average viable cells.

Figure 17. The effects of pCCOM (green) and pCCOMB (black) on *E. coli* JM103 Δ *umuC* strains following UV treatment. The cells were treated with UV for a period of 2 minutes and aliquots were taken every 15 seconds. The cells were then grown overnight in the absence of light and plated onto LB and LB + rifampicin. The negative control (red) is deficient in *mucA/B* and the positive control (blue) contains a plasmid encoding *mucA/B*. The number of induced rifampicin mutants are expressed as a ratio of average rifampicin mutants/average viable cells.

Table 9. Nitrofurazone reductase assay for TA1538 (frameshift) derivatives. The results are derived from 2 independent inoculations. The protein concentrations were determined by the method of Lowry *et al.* (1951) using BSA as a standard. The results are expressed as $\mu\text{mole/min/mg protein} \pm \text{standard deviation}$.

Strain	Plasmid	Description	Specific Activity ($\mu\text{mol/min/mg}$) $\pm$ S.D.
TA1538	none	wild-type	17.6 $\pm$ 1.2
CFT500	pBR322	cloning vector	42.8 $\pm$ 2.4
CFT501	pGW1700	<i>mucA/B</i>	14
TA98	pKM101	<i>mucA/B</i>	14.5 $\pm$ 2.0
TA98NR	pKM101	<i>mucA/B</i>	1.0
YG1021	pYG216	<i>cnr</i>	17.5 $\pm$ 1.5
YG1024	PYG219	<i>OAT</i>	35.6 $\pm$ 13.7
YG1041	pYG233	<i>cnr</i> , <i>OAT</i>	1540 $\pm$ 185
CFT502	pBR322A	<i>nfsA</i>	1860 $\pm$ 221
CFT503	pBR322B	<i>nfsB</i>	17.6 $\pm$ 0.6
CFT504	pYG219	<i>OAT</i>	15.3 $\pm$ 1.6
CFT505	pCCOA	<i>OAT</i> , <i>nfsA</i>	3370 $\pm$ 291
CFT506	pCCOB	<i>OAT</i> , <i>nfsB</i>	10 $\pm$ 2
CFT507	pCCOAB	<i>OAT</i> , <i>nfsA</i> , <i>nfsB</i>	964 $\pm$ 110
CFT508	pCCOM	<i>OAT</i> , <i>mucA/B</i>	35.6 $\pm$ 12.1
CFT509	pCCOMA	<i>OAT</i> , <i>nfsA</i> , <i>mucA/B</i>	1950 $\pm$ 95.7
CFT510	pCCOMB	<i>OAT</i> , <i>nfsB</i> , <i>mucA/B</i>	11.1 $\pm$ 0.1
CFT511	pCCOMBA	<i>OAT</i> , <i>nfsA</i> , <i>nfsB</i> , <i>mucA/B</i>	5740 $\pm$ 1160
CFA504	pYG213	<i>OAT</i>	21.5 $\pm$ 5.7
CFA505	p213A	<i>OAT</i> , <i>nfsA</i>	145 $\pm$ 43.5
CFA506	p213B	<i>OAT</i> , <i>nfsB</i>	18.7 $\pm$ 0.1
CFA507	p213AB	<i>OAT</i> , <i>nfsA</i> , <i>nfsB</i>	22.2 $\pm$ 4.4
CFA508	p213M	<i>OAT</i> , <i>mucA/B</i>	23.1 $\pm$ 2.2
CFA509	p213MA	<i>OAT</i> , <i>nfsA</i> , <i>mucA/B</i>	280 $\pm$ 34
CFA510	p213MB	<i>OAT</i> , <i>nfsB</i> , <i>mucA/B</i>	13.6 $\pm$ 5.1
CFA511	p213MBA	<i>OAT</i> , <i>nfsA</i> , <i>nfsB</i> , <i>mucA/B</i>	347 $\pm$ 56.4

Table 10. Nitrofurazone reductase assay for TA1535 (base substitution) derivatives. The results are derived from 2 independent inoculations. The protein concentrations were determined by the method of Lowry *et al.* (1951) using BSA as a standard. The results are expressed as $\mu\text{mole/min/mg protein} \pm \text{standard deviation difference}$.

Strain	Plasmid	Description	Specific Activity ($\mu\text{mol}/\text{min}/\text{mg}$) $\pm$ S.D.
TA1535	none	wild-type	37.2 ± 2.8
CBT400	pBR322	cloning vector	16.3 ± 0.4
CBT401	pGW1700	<i>mucA/B</i>	28.1 ± 1.0
TA100	pKM101	<i>mucA/B</i>	27 ± 5.6
YG1026	pYG216	<i>cnr</i>	863 ± 3.7
YG1029	PYG219	<i>OAT</i>	19.2 ± 2.0
YG1042	pYG233	<i>cnr, OAT</i>	672 ± 30.8
CBT402	pBR322A	<i>nfsA</i>	2380 ± 102
CBT403	pBR322B	<i>nfsB</i>	33
CBT404	pYG219	<i>OAT</i>	26.4 ± 2.4
CBT405	pCCOA	<i>OAT, nfsA</i>	3610 ± 708
CBT406	pCCOB	<i>OAT, nfsB</i>	36.2 ± 8.4
CBT407	pCCOAB	<i>OAT, nfsA, nfsB</i>	4800 ± 1200
CBT408	pCCOM	<i>OAT, mucA/B</i>	32.7 ± 1.6
CBT409	pCCOMA	<i>OAT, nfsA, mucA/B</i>	3830 ± 1590
CBT410	pCCOMB	<i>OAT, nfsB, mucA/B</i>	33.4 ± 1.9
CBT411	pCCOMBA	<i>OAT, nfsA, nfsB, mucA/B</i>	5550 ± 667
CBA404	pYG213	<i>OAT</i>	37.2 ± 7.4
CBA405	p213A	<i>OAT, nfsA</i>	94.5 ± 0.3
CBA406	p213B	<i>OAT, nfsB</i>	33.1 ± 1.9
CBA407	p213BA	<i>OAT, nfsA, nfsB</i>	120 ± 8.5
CBA408	p213M	<i>OAT, mucA/B</i>	36.6 ± 3.4
CBA409	p213MA	<i>OAT, nfsA, mucA/B</i>	248 ± 244
CBA410	p213MB	<i>OAT, nfsB, mucA/B</i>	35.2 ± 2.5
CBA411	p213MBA	<i>OAT, nfsA, nfsB, mucA/B</i>	323 ± 45.1

Figure 18. A printout from the Ultrospec 3000 for strain CFT509 during the NFR assay. Reaction rates were determined by Ultrospec from the linear portion of the reaction. The result was divided by the protein concentration and then divided by 2 since 2 μ l of protein extract was used. The molar extinction coefficient for NF at 400 nm is 12,960 $M^{-1}cm^{-1}$. The protein concentrations were determined by the method of Lowry *et al.* (1951) using BSA as a standard.

resistant) had much higher enzyme activities than their counterparts encoding ampicillin resistance markers. In addition, strains containing *nfsA* constructs had higher enzyme activities when compared to *nfsB* constructs. Moreover, the highest enzyme activity reported came from strains containing pCCOMBA. The lowest enzyme activity came from TA98NR which is consistent with the fact that this strain is deficient in both *cnr* and *snrA*. Enzyme activity in the previously established strain YG1026 containing the *cnr* construct were much higher than in strains containing only *nfsB* constructs, but lower than in strains containing only *nfsA* constructs. In most cases, the presence of *OAT* and *mucA/B* did not significantly alter the nitroreductase activity of the strain. A comparison of enzyme activities between wild-type TA1535 and wild-type TA1538 showed that TA1535 had approximately 2.1-fold more endogenous nitroreduction than TA1538. Similarly, several other TA1535 strains, also showed this trend when compared to their TA1538 counterparts.

3.5 Mutagenicity testing

Several of the new tester strains possessing elevated metabolic functions toward nitro substituted compounds were evaluated using the standard Ames mutagenicity test (as described in section 2.13). These included the frameshift strains CFA504, CFA507, CFA508, CFA509, CFA510, CFA511, CFT500, CFT501, CFT504, CFT507, CFT508, CFT509, CFT510, CFT511, and the base substitution strains CBA404, CBA407, CBA408, CBA409, CBA410, CBA411, CBT400, CBT401, CBT404, CBT407, CBT408, CBT409, CBT410, and CBT411. The battery of chemicals tested included six typical nitrosubstituted compounds: 1-NP, 1,6-DNP, 2-NF, 1-NN, NF, and 4-NQO, and 2 non-

nitrosubstituted compounds, ICR-191 and EMS , as controls. Figure 19 illustrates a typical mutagenicity test result for strain CFA504 with 1,6-DNP, compared to a plate of spontaneous revertants.

Since the chemicals tested vary in their mutagenicity it was important to find a range of doses that would yield a suitable dose response curve. For example, chemicals such as 1,6-DNP exerted a high mutagenicity at extremely low doses, while a large dose was needed to observe mutagenicity in strains treated with 2-NN. The doses were determined by consulting several previous published papers by Hagiwara *et al.* (1993) and Watanabe *et al.* (1989; 1990). Some preliminary experiments were also performed to test the sensitivity of the new strains and to confirm that the doses chosen were adequate for testing.

Each dose was done in triplicate and each plate was counted three times; a mean number of revertants was calculated. The induced revertants per plate were calculated by subtracting the mean spontaneous revertants from each dose and then dividing this number by the number of nanomoles of mutagen to yield the number of induced His⁺ revertants per nanomole (data presented in Tables 11-16). The standard deviation difference (S.D._(Diff)) was calculated for each dose according to the following formula:

$$S.D_{(Diff)} = [(S.D_{obs})^2 + (S.D_{spont})^2]^{1/2} \text{ where;}$$

S.D_{obs} is the standard deviation of the observed result

S.D._{spont} is the standard deviation of the spontaneous revertants

The standard deviation difference was then divided by the number of nanomoles of mutagen for each dose.

Figure 19. A typical mutagenicity test result depicting His⁺ revertants for strain CFA504 and 1,6-DNP (2). Spontaneous His⁺ revertants are shown for CFA504 in (1). The dark background was created during scanning.

1 ►

2 ►

Table 1 1. Mutagenicity testing results for several previously established *S. typhimurium* frameshift tester strains. Some possess different metabolic capacities toward nitrosubstituted compounds. The results are taken from 3 plates for each dose and each plate was counted 3 times using an automated plate counter. The mutagenicity is expressed as induced revertants per nanomole $\pm$ the standard deviation difference

^A The number of His⁺ revertants per μ mole is indicated.

^B No response was detected.

Induced His ⁺ revertants per nanomole				
Chemical	TA98 pKM101 <i>mucA/B</i>	YG1021 pKM101, pYG216 <i>mucA/B, cnr</i>	YG1024 pKM101, pYG219 <i>mucA/B, OAT</i>	YG1041 pKM101, pYG233 <i>mucA/B, OAT, cnr</i>
2-NF	45 ± 19	410 ± 32	970 ± 88	320 ± 49
1-NP	910 ± 430	137,000 ± 34,900	15,500 ± 2,045	130,000 ± 3,700
1,6-DNP	51,000 ± 9,900	5,900,000 ± 330,000	5,065,000 ± 1,390,000	8,410,000 ± 5,560,000
NF	6 ± 2	8 ± 5	30 ± 3	5 ± 2
2-NN	3 ± 1	8 ± 10	3 ± 1	50 ± 9
4-NQO	203 ± 109	694 ± 272	340 ± 26	320 ± 102
ICR-191	9 ± 4	10 ± 2	30 ± 1	30 ± 4
^a EMS	^b NR	^b NR	0.7 ± 0.1	0.9 ± 0.1

Table 12. Mutagenicity testing results for several newly constructed *S. typhimurium* frameshift (ampicillin) tester strains possessing different metabolic capacities toward nitrosubstituted compounds. The results are taken from 3 plates for each dose and each plate was counted 3 times using an automated plate counter. The mutagenicity is expressed as induced revertants per nanomole $\pm$ the standard deviation difference.

^A The number of His⁺ revertants per μ mole is indicated.

Induced His ⁺ revertants per nanomole				
Chemical	CFA504 pYG213 OAT	CFA507 p213AB OAT, <i>nf</i> sA, <i>nf</i> sB	CFA508 p213M OAT, <i>mucA</i> /B	CFA509 p213MA OAT, <i>mucA</i> /B, <i>nf</i> sA
2-NF	646 ± 113	851 ± 183	332 ± 192	1,900 ± 81
1-NP	15,500 ± 2,810	4,073 ± 344	6,560 ± 1,580	175,000 ± 51,800
1,6-DNP	1,670,000 ± 131,000	354,000 ± 25,600	4,430,000 ± 144,000	6,240,000 ± 399,000
NF	44 ± 24	2 ± 10	330 ± 66	30 ± 4
2-NN	20 ± 4	1 ± 1	4 ± 3	30 ± 7
4-NQO	3,610 ± 2,390	180 ± 74	340 ± 140	519 ± 135
ICR-191	34 ± 12	20 ± 3	40 ± 6	20 ± 1
^a EMS	0.2 ± 0.2	0.5 ± 0.7	2 ± 1	2 ± 1

Table 12. Continued

Induced His⁺ revertants per nanomole		
Chemical	CFA510 p213MB <i>OAT, mucA/B, nfsB</i>	CFA511 p213MBA <i>OAT, mucA/B, nfsB, nfsA</i>
2-NF	340 ± 58	1,720 ± 126
1-NP	27,900 ± 3,450	146,000 ± 135,000
1,6-DNP	63,900 ± 21,900	2,250,000 ± 451,000
NF	20 ± 20	40 ± 6
2-NN	2 ± 1	20 ± 10
4-NQO	347 ± 102	818 ± 133
ICR-191	30 ± 4	30 ± 6
^AEMS	1 ± 1	2 ± 1

Table 13. Mutagenicity testing results for several newly constructed *S. typhimurium* frameshift (tetracycline) tester strains possessing different metabolic capacities toward nitrosubstituted compounds. The results are taken from 3 plates for each dose and each plate was counted 3 times using an automated plate counter. The mutagenicity is expressed as induced revertants per nanomole $\pm$ the standard deviation difference.

^A The number of His⁺ revertants per μ mole is indicated.

^B No response was detected.

Induced His ⁺ revertants per nanomole						
Chemical	CFT500 pBR322 None	CFT501 pGW1700 <i>mucA/B</i>	CFT504 pYG219 <i>OAT</i>	CFT507 pCCOAB <i>OAT, nfsA, nfsB</i>	CFT508 pCCOM <i>OAT, mucA/B</i>	CFT509 pCCOMA <i>OAT, mucA/B, nfsA</i>
2-NF	2 ± 6	10 ± 2	340 ± 38	420 ± 54	350 ± 72	1,930 ± 401
1-NP	^a NR	1,050 ± 237	7,440 ± 3,760	18,000 ± 1,400	6,240 ± 829	227,000 ± 13,900
1,6-DNP	563 ± 1,310	197,000 ± 43,500	427,000 ± 89,400	1,390,000 ± 89,500	572,000 ± 86,600	2,620,000 ± 781,000
NF	18 ± 16	40 ± 20	3 ± 2	60 ± 6	210 ± 24	120 ± 68
2-NN	1 ± 1	4 ± 1	6 ± 2	20 ± 4	10 ± 2	30 ± 10
4-NQO	16 ± 11	1,470 ± 196	170 ± 39	3,210 ± 1,230	3,340 ± 468	1640 ± 169
ICR-191	9 ± 30	0.4 ± 0.5	5 ± 2	20 ± 6	30 ± 2	9 ± 2
^a EMS	7 ± 1	0.6 ± 0.4	0.2 ± 0.2	^b NR	7 ± 1	2 ± 2

Table 13. Continued

Induced His ⁺ revertants per nanomole		
Chemical	CFT510 pCCOMB <i>OAT, mucA/B, nfsB</i>	CFT511 pCCOMBA <i>OAT, mucA/B, nfsB, nfsA</i>
2-NF	440 ± 81	3570 ± 364
1-NP	36,500 ± 3,470	50,200 ± 7,380
1,6-DNP	1,270 ± 1,990	6,640,000 ± 699,000
NF	30 ± 10	100 ± 5
2-NN	3 ± 1	30 ± 5
4-NQO	471 ± 144	994 ± 127
ICR-191	30 ± 2	30 ± 6
^A EMS	0.3 ± 0.3	3 ± 3

Table 14. Mutagenicity testing results for several previously established *S. typhimurium* base substitution tester strains. Some possess different metabolic capacities toward nitro substituted compounds. The results are taken from 3 plates for each dose and each plate was counted 3 times using an automated plate counter. The mutagenicity is expressed as induced revertants per nanomole $\pm$ the standard deviation difference.

^A The number of His⁺ revertants per μ mole is indicated.

Induced His ⁺ revertants per nanomole				
Chemical	TA100 pKM101 <i>mucA/B</i>	YGI026 pKM101, pYG216 <i>mucA/B, cnr</i>	YGI029 pKM101, pYG219 <i>mucA/B, OAT</i>	YGI042 pKM101, pYG233 <i>mucA/B, OAT, cnr</i>
2-NF	30 ± 8	210 ± 77	77 ± 34	66 ± 28
1-NP	260 ± 24	810 ± 83	898 ± 128	18,000 ± 3,000
1,6-DNP	16,700 ± 5,390	392 ± 202	9,100 ± 1,500	739,000 ± 148,000
NF	220 ± 24	300 ± 60	20 ± 5	260 ± 29
2-NN	2 ± 1	8 ± 2	0.5 ± 0.1	120 ± 26
4-NQO	1,500 ± 35	1,460 ± 211	287 ± 105	1480 ± 346
ICR-191	30 ± 2	21 ± 12	20 ± 1	30 ± 7
^a EMS	4 ± 2	7 ± 1	5 ± 2	5 ± 5

Table 15. Mutagenicity testing results for several newly constructed *S. typhimurium* base substitution (ampicillin) tester strains possessing different metabolic capacities toward nitrosubstituted compounds. The results are taken from 3 plates for each dose and each plate was counted 3 times using an automated plate counter. The mutagenicity is expressed as induced revertants per nanomole $\pm$ the standard deviation difference.

^A The number of His⁺ revertants per μ mole is indicated.

^B No response was detected.

Chemical	Induced His ⁺ revertants per nanomole			
	CBA404 pYG213 OAT	CBA407 p213AB OAT, <i>nfsA</i> , <i>nfsB</i>	CBA408 p213M OAT, <i>mucA/B</i>	CBA409 p213MA OAT, <i>mucA/B</i> , <i>nfsA</i>
2-NF	1 ± 1	1 ± 2	56 ± 27	2,000 ± 200
1-NP	26 ± 63	52 ± 43	9,830 ± 1,990	13,100 ± 1,880
1,6-DNP	38 ± 220	^b NR	1,050,000 ± 349,000	679,000 ± 35,900
NF	6 ± 20	6 ± 7	240 ± 31	390 ± 30
2-NN	7 ± 2	30 ± 3	20 ± 9	60 ± 3
4-NQO	10 ± 9	54 ± 72	3,390 ± 1,830	8,690 ± 741
ICR-191	0.5 ± 0.2	0.4 ± 0.5	30 ± 7	30 ± 6
^a EMS	9 ± 3	5 ± 1	7 ± 2	9 ± 1

Table 15. Continued

Induced His ⁺ revertants per nanomole		
Chemical	CBA410 p213MB <i>OAT, mucA/B, nfsB</i>	CBA411 p213MBA <i>OAT, mucA/B, nfsB, nfsA</i>
2-NF	86 ± 17	629 ± 156
1-NP	8,540 ± 3,190	7,560 ± 4,710
1,6-DNP	362,000 ± 181,000	92,000 ± 26,000
NF	280 ± 15	1,020 ± 173
2-NN	10 ± 1	30 ± 5
4-NQO	4,040 ± 1,160	2,220 ± 335
ICR-191	16 ± 12	20 ± 9
^Λ EMS	9 ± 2	3 ± 2

Table 16. Mutagenicity testing results for several newly constructed *S. typhimurium* base substitution (tetracycline) tester strains possessing different metabolic capacities toward nitrosubstituted compounds. The results are taken from 3 plates for each dose and each plate was counted 3 times using an automated plate counter. The mutagenicity is expressed as induced revertants per nanomole $\pm$ the standard deviation difference.

^A The number of His⁺ revertants per μ mole is indicated.

^B No response was detected.

Induced His ⁺ revertants per nanomole						
Chemical	CBT400 pBR322 None	CBT401 pGW1700 <i>mucA/B</i>	CBT404 pYG219 <i>OAT</i>	CBT407 pCCOAB <i>OAT, nfsA, nfsB</i>	CBT408 pCCOM <i>OAT, mucA/B</i>	CBT409 pCCOMA <i>OAT, mucA/B, nfsA</i>
2-NF	20 ± 3	23 ± 11	^b NR	6 ± 13	300 ± 50	2,300 ± 170
1-NP	200 ± 5	120 ± 24	18 ± 160	74 ± 330	3,150 ± 515	8,200 ± 220
1,6-DNP	^b NR	510 ± 330	30,500 ± 47,700	170 ± 120	562,000 ± 104,000	5,590,000 ± 513,000
NF	5 ± 3	210 ± 17	28 ± 23	8 ± 3	160 ± 12	260 ± 14
2-NN	0.2 ± 0.1	2 ± 1	7 ± 1	30 ± 3	10 ± 1	60 ± 4
4-NQO	180 ± 46	2,680 ± 129	140 ± 33	56 ± 68	4,210 ± 1,830	3900 ± 1,200
ICR-191	7 ± 1	19 ± 14	5 ± 6	16 ± 18	30 ± 6	27 ± 17
^a EMS	0.4 ± 0.1	6 ± 2	5 ± 2	5 ± 1	5 ± 1	6 ± 2

Table 16. Continued

Induced His ⁺ revertants per nanomole		
Chemical	CBT410 pCCOMB <i>OAT, mucA/B, nfsB</i>	CBT411 pCCOMBA <i>OAT, mucA/B, nfsB, nfsA</i>
2-NF	47 ± 13	1,450 ± 187
1-NP	5,390 ± 2010	8,308 ± 739
1,6-DNP	144,000 ± 120,000	3,290,000 ± 9,170,000
NF	300 ± 50	350 ± 61
2-NN	35 ± 31	170 ± 21
4-NQO	4,200 ± 1,100	5,920 ± 1,820
ICR-191	9 ± 1	30 ± 4
^A EMS	8 ± 2	8 ± 3

3.5.1 Mutagenicity in the previously established frameshift tester strains

The previously established frameshift tester strains (Watanabe *et al.*, 1989; 1990; Hagiwara *et al.*, 1993) are listed in Table 11. When compared to TA98, both YG1041 and YG1021 showed high mutagenicity for several potent frameshift compounds such as 1-NP and 1,6-DNP. In addition, the presence of the *cnr* gene increased the number of revertants per nanomole dramatically for compounds such as 2-NF, 1-NP, 1,6-DNP, and 4-NQO. For YG1024 the presence of the *OAT* gene increased the number of revertants for both 1-NP and 1,6-DNP. The chemicals which are known to induce primarily base substitutions i.e. 2-NN, NF, 4-NQO, and EMS did not significantly increase the number of revertants in these frameshift strains. Similarly, the non-nitro compounds showed little increase in the number of revertants.

A pairwise comparison was made between the frameshift strain YG1041 and the base substitution strain YG1042 for all chemicals tested (Table 17). The ratio of the mutagenicity test results for these strains indicate that 2-NF, 1-NP, and 1,6-DNP preferentially induce frameshifts at *hisD3052* as compared to base substitutions at *hisG46*.

3.5.2 Mutagenicity in the newly constructed frameshift tester strains (CFA-series and CFT-series)

The newly constructed frameshift strains harbouring both ampicillin resistant markers (CFA-series) and tetracycline resistant markers (CFT-series) are listed in Tables 12 and 13. For the nitrosubstituted compounds the presence of either *nfsA* or *nfsB* did cause a stimulation in the number of revertants per nanomole when compared to strains

Table 17. A pairwise comparison between mutation frequencies in frameshift strains CFT511, CFA511, and YG1041 as compared to their base substitution counterparts CBT411, CBA411, and YG1042.

Chemicals	Frameshift mutations:Base substitution mutations		
	CFT511:CBT411	CFA511:CBA411	YG1041:YG1042
	pCCOMBA	p213MBA	pKM101; pYG233
2-NF	3	3	5
1-NP	6	19	7
1,6-DNP	2	25	11
NF	0.3	0	0
2-NN	0.2	0.7	0.4
4-NQO	0.2	0.4	0.2
ICR-191	1	2	1
EMS	0.4	0.7	0.2

without these two genes. The most sensitive strains toward the frameshift compounds 1,6-DNP, 1-NP, and 2-NF were CFA509, CFT509, CFA511, and CFT511. Chemicals such as 2-NN and NF, which are known to induce primarily base substitutions, did not cause a notable increase in the number of revertants for most strains. However, 4-NQO did show an increase in the number of revertants. The presence of the *OAT* gene in strain CFA504 caused a substantial stimulation in the number of revertants for most compounds especially 1,6-DNP, when compared to CFT500. The non-nitro compounds were not affected by the increased cellular nitroreduction capacity.

A pairwise comparison was made between frameshift strains within their group which differ only with respect to whether or not they contain *nfsA* on the plasmid; this allows us to estimate the mutagenic effects of plasmid-borne *nfsA* on the battery of test chemicals (Table 18). For example, comparisons were made between the mutagenicity test results for strains CFA511 [p213MBA (*mucA/B*, *OAT*, *nfsB*, *nfsA*)] and CFA510 [p213MB (*mucA/B*, *OAT*, *nfsB*)], and between CFT511 [pCCOMBA (*mucA/B*, *OAT*, *nfsB*, *nfsA*)] and CFT510 [pCCOMBA (*mucA/B* *OAT* *nfsB*)]. The ratios of the two matched comparisons, CFA511/CFA510 and CFT511/CFT510, were averaged to give CF511:510 and are shown in Table 18. It is clear from this data, that NfsA preferentially stimulates frameshift mutations when 2-NF, 1,6-DNP, and 2-NN are used as test chemicals. Interestingly, 1-NP, a known frameshift mutagen, is not stimulated significantly by NfsA.

The mutagenic effect of NfsB was analyzed in the same way as described above for NfsA. The comparisons were made within matched strain pairs CFA510/CFA508 and CFT510/CFT508. The average values of these comparisons are illustrated in Table

Table 18. A pairwise comparison between frameshift strains (CFT-series and CFB-series) to illustrate the effects of plasmid-borne NfsA, NfsB, NfsA/NfsB, and MucA/B on chemically induced frameshift mutations.

Chemicals	Effect of <i>nfsA</i> Average CF511:510	Effects of <i>nfsB</i> Average CF510:508	Effect of <i>NfsA/NfsB</i> Average CF511:508	Effect of <i>MucA/B</i> Average CF511:507
2-NF	7	1	8	5
1-NP	3	5	15	19
1,6-DNP	2642	0.01	6	6
NF	2	0.1	0.3	10
2-NN	10	0.4	4	9
4-NQO	2	0.6	1	2
ICR-191	1	1.0	0.8	2
EMS	6	0.3	0.7	4

18. This data suggests that *nfsB*, the minor nitroreductase, does not stimulate frameshift mutations induced by most nitrosubstituted chemicals, the only exception being 1-NP. In fact, NfsB appears to exert an inhibitory effect on mutagenesis induced by most of the nitrosubstituted chemicals, most noticeably 1,6-DNP.

The effect of having both NfsA and NfsB encoded by plasmid-borne genes was examined using mutagenicity data from CFA511/CFA508 and CFT511/CFT508 matched pairs. The average values of these comparisons are illustrated in Table 18. Frameshift mutations induced by 2-NF, 1-NP, 1,6-DNP, and 2-NN were all stimulated by the presence of both the major and minor nitroreductase genes. In addition, the mutagenicity for 1-NP seems to be significantly higher when both nitroreductase genes are present than just NfsB alone. The possible inhibitory effect of NfsB on 1,6-DNP is evident in this data set.

The effect of plasmid-borne MucA/B on frameshift mutagenesis was also examined. The matched strain pairs used in this comparison were CFA511/CFA507 and CFT511/CFT507. The average values of these comparisons are illustrated in Table 18. The presence of MucA/B appeared to stimulate frameshift mutagenesis for all chemicals, particularly 2-NF, 1-NP, 1,6-DNP, NF, and 2-NN.

A final comparison was made between the frameshift strains and base substitution strains which contain the same plasmid: CFT511 vs. CBT411 and CFA511 vs. CBA411. The values are shown in Table 19, and support the earlier observation (section 3.5.1) that 2-NF, 1-NP, and 1,6-DNP preferentially induce frameshift mutations at *hisD3052* as compared to base substitution mutations at *hisG46*.

Table 19. A pairwise comparison between base substitution strains (CBT-series and CBA-series) to illustrate the effects of plasmid-borne NfsA, NfsB, NfsA/NfsB, and MucA/B on chemically induced base substitution mutations.

Chemicals	Effect of NfsA Average CB411:410	Effects of NfsB Average CB410:408	Effect of NfsA/NfsB Average CB411:408	Effect of MucA/B Average CB411:407
2-NF	19	1	8	435
1-NP	1	3	1	129
1,6-DNP	12	0.2	3	55,678
NF	2	0.7	3	107
2-NN	4	0.5	9	4
4NQO	1	0.7	1	73
ICR-191	2	0.9	0.8	25
EMS	0.7	0.7	0.8	1

3.5.3 Mutagenicity in the previously established base substitution tester strains

Mutagenicity in previously established base substitution strains are listed in Table 14. The presence of the *cnr* gene in strains YG1026 and YG1042 caused a marked stimulation in the number of revertants, when compared to YG1029, for compounds NF, 2-NN, and 4-NQO. Interestingly, the frameshift compound 1-NP also induced more base substitutions in YG1026, YG1029, and YG1042 when compared to TA100. The frameshift compound 1,6-DNP induced a marked increase in revertants only in strain YG1042. Overall, YG1042 was the most sensitive base substitution strain tested among the previously established strains. Both EMS and ICR-191 gave a consistent number of revertants for all strains; however, the numbers for EMS were notably higher than those of the frameshift strains, as expected for this base substitution mutagen.

3.5.4 Mutagenicity in the newly constructed base substitution tester strains (CBA-series and CBT-series)

The base substitution strains conferring ampicillin and tetracycline resistance are listed in Tables 15 and 16. The increased metabolic capacities of these strains caused a marked increase in the number of revertants, but only in strains containing MucA/B. Strains lacking MucA/B, such as CBT400, CBT407, CBA404 and CBA407, all showed little response to the chemical treatment. Strain CBT404, which lacks MucA/B, did show an appreciable increase in the number of revertants for 1,6-DNP only. The most sensitive strains towards most nitro compounds were CBA409, CBA411, CBT409, and CBT411. The presence of OAT and MucA/B in CBA408 and CBT408 also caused the number of revertants to increase substantially when compared to CBT400.

A pairwise comparison was made between base substitution strains to illustrate the mutagenic effects of having plasmid-borne NfsA (Table 19). The comparisons were made by comparing mutagenicity test results for strains that differ only by NfsA. The comparisons were done using CBA411/CBA410 and CBT411/CBT410 matched strain pairs. The average values are shown in Table 19. Base substitution mutagenesis seems to be significantly stimulated by NfsA when the cells are treated with 2-NF, 1,6-DNP, 2-NN, and, to a lesser extent, NF. This is consistent with the results observed in the frameshift tester strains.

The effect of NfsB in promoting base substitution mutagenesis induced by nitrosubstituted chemicals can be analyzed in the same way as for NfsA. The comparisons were made within the matched strain pairs CBA410/CBA408 and CBT410/CBT408. The average values are shown in Table 19. NfsB appears to only stimulate base substitutions in cells treated with 1-NP. The remaining chemicals do not appear to be affected by the presence of NfsB.

The effect of both NfsA and NfsB on base substitution mutagenesis was evaluated using the matched strain pairs CBA411/CBA408 and CBT411/CBT408. The average values of these comparisons are shown in Table 19. The presence of both the major and minor nitroreductase genes appears to stimulate base substitution mutagenesis in cells treated with 2-NF, 1,6-DNP, NF, and 2-NN. The inhibitory effect of NfsB, observed in several of the frameshift tester strains, was not observed with the base substitution strains expressing both NfsA and NfsB.

The effect of MucA/B on base substitution mutagenesis was examined using CBA411/CBA407 and CBT411/CBT407 as matched pairs for comparative purposes.

The average values of these comparisons are shown in Table 19. The presence of MucA/B significantly enhanced base substitution mutagenesis for most chemicals, but the results were most striking for 2-NF, 1-NP, 1,6-DNP, NF, 4-NQO, and ICR-191. Interestingly, those chemicals that exhibit the highest ratios of frameshifts/base substitutions, 2-NF, 1-NP and 1,6-DNP (Table 17) are those whose base substitution activity appears to be most dependent on the presence of MucA/B.

4.0 DISCUSSION

Bacterial mutagenicity assays are the most common STTs used in genotoxicology testing. Advances in molecular biology have made it possible to develop bacterial tester strains with enhanced metabolic properties. To date, the focus has been to design multi-copy plasmids that contain genes encoding key metabolic proteins, and introduce them into conventional Ames tester strains (Watanabe *et al.*, 1989; 1990; Hagiwara *et al.*, 1993; Josephy *et al.*, 1997; 1999; Constable *et al.*, 1999).

The additional metabolic capacity enhances the ability of the tester strain to bioactivate mutagens to DNA reactive species. In the past, all bacterial reversion assays have utilized a two plasmid system i.e. pKM101 and a plasmid bearing metabolic genes, which were maintained by two different antibiotics. The work reported here describes several new *S. typhimurium* tester strains that harbour a single multi-copy plasmid containing all the necessary genes for bioactivation and mutagenesis of nitrosubstituted compounds. The single plasmid system is an attractive alternative to the two plasmid system, as only one antibiotic is required for plasmid maintenance. In addition, each plasmid was constructed in two forms. One form was ampicillin resistant (p213-constructs) and the other form was tetracycline resistant (pCC-constructs) (see plasmid maps Figs. 8-12). The two different forms of each plasmid may provide some flexibility when used in a bacterial mutagenicity assay that already relies on a plasmid. For example, there are several current initiatives that are attempting to develop plasmids expressing cytochrome P450 (Josephy *et al.*, 1997; 1999; Constable *et al.*, 1999). Bacterial mutagenicity tester strains with enhanced reductive and oxidative capacity may be sensitive to a broader range of mutagens.

4.1 The activity of cloned *nfsA* and *nfsB*

The expression of cloned nitroreductase genes in a nitroreductase deficient host had deleterious effects on the cells when plated on nitrofurazone media. Bacterial cells containing the major nitroreductase gene *nfsA* by itself, or in combination with *nfsB* showed large killing zones when compared to cells lacking *nfsA* (Fig. 13 and Fig. 14). In fact, most of the ampicillin constructs showed a stepwise increase in their killing zones that coincided with the addition of a nitroreductase gene. The large killing zones can be attributed to the increase in reductive activation of nitrofurazone, which has strong antimicrobial qualities. When both *nfsB* and *nfsA* were on the same plasmid the killing zones were among the highest reported (Fig. 14). A statistical comparison between the size of the killing zones of cells containing *nfsA* plasmids to those lacking *nfsA* always revealed a statistically significant result ($0.05 < P < 0.001$). Cells containing plasmids lacking nitroreductase genes showed smaller killing zones when compared to cells with increased nitroreduction capacity. Plasmids containing the *cnr* gene, the minor nitroreductase isolated from *S. typhimurium*, exhibited similar killing effects as plasmids containing *nfsA*, the major *E. coli* nitroreductase, when *E. coli* containing these plasmids were plated on nitrofurazone.

Following verification that the cloned nitroreductases were functional in *E. coli* the plasmids were transformed into *S. typhimurium* TA1535 and TA1538. The effect the cloned nitroreductase genes had on the reduction of nitrofurazone was measured by the decrease in absorbance of nitrofurazone at 400 nm. The wild-type strains, TA1535 and TA1538 were previously thought to be isogenic with regard to their nitroreductase activity (Yamada *et al.*, 1997). Recent work done by Bouramandi *et al.* (in preparation)

provides strong evidence that the *snrA* gene, a homolog of *E. coli nfsA*, has been deleted in TA1538. Bouramandi *et al.* (in preparation) provides compelling evidence that these two strains are, in fact, genetically different with respect to their nitroreductase genes. Yamada *et al.* (1997) reported a 4.6-fold higher NFR activity for TA1535 than TA1538 in a similar nitrofurazone reductase assay to the one performed in this work. The NFR activity for TA1535 in this work was approximately 2.1-fold higher than TA1538 (Tables 9 and 10). The increase in observed cellular nitroreductase levels in TA1535, as compared to TA1538, may be attributed to the fact that in TA1535 the majority of chromosomally encoded Type I nitroreductase activity is derived from SnrA and Cnr, while in TA1538 only Cnr is functional.

TA1538 and TA1535 derivatives harbouring plasmids containing *nfsA* showed high levels of nitroreductase activity (Tables 9 and 10). This effect was more pronounced with the tetracycline resistant constructs than with those harbouring the ampicillin resistant markers. These results are supported by biochemical studies showing that *nfsA* is much more efficient at reducing nitrofurazone than *nfsB* (Zenno *et al.*, 1996a; Yamada *et al.*, 1997; Whiteway *et al.*, 1998). The extremely high level of NFR activity in cells containing the plasmid-borne nitroreductases is probably due to the gene dosage effect of the cloned nitroreductase genes on multicopy vectors. The highest nitroreductase values came from cells containing both the major and minor *E. coli* nitroreductases i.e. pCCOMBA. For strains that were extremely efficient at reducing nitrofurazone, a 10-100-fold dilution was required to achieve a linear relationship (Fig. 18). Conversely TA98NR, which acted as a negative control, is deficient in *cnr* and *snrA* and showed little if any detectable nitroreductase activity (Table 9). Furthermore, strains

harbouring plasmids with only *OAT* and *mucA/B* also yielded low specific activities since their gene products do not contribute to the reduction of nitrofurazone.

All the ampicillin resistant constructs showed much lower specific nitroreductase activities in cellular extracts when compared to their tetracycline resistant counterparts. The reason(s) why this occurred is not known, but at least two explanations are possible.

1) It could be that the plasmids are maintained at different copy numbers within the cell. This would effect the nitroreductase level present in the cell extracts. 2) Tetracycline may induce the *marRAB* regulon which regulates the *soxRS* regulon of which *nfsA* is a member (Liochev *et al.*, 1999). This may lead to upregulation of nitroreductase within the cell. *In vivo*, differences in activity may also be related to the different modes of action of the two antibiotics. Tetracycline resistance is maintained by a membrane bound protein pump which pumps the tetracycline out of the cell. Conversely, ampicillin resistance is maintained by β -lactamase which degrades the ampicillin over time before it affects the synthesis of the bacterial cell wall. It is possible that the permeability of the membrane is altered in such a way by the tetracycline pump that it would favour the uptake of nitrofurazone. The increased uptake of nitrofurazone would provide more substrate for the nitroreductase enzymes.

4.2 Mutagenicity Testing

For all mutagens tested a linear portion of the dose response curve was apparent and the number of revertants per plate was taken from this region of the curve. If the results were determined from the descending portion of the curve, the results would be misleading as this is where toxicity rather than mutagenicity is observed. On each day of

testing, the chemicals were diluted in fresh DMSO and the tester strains were grown for a 12 hour growth period. An aliquot of the cell culture was also plated onto LB+tetracycline or ampicillin to determine the titer of the initial culture. The Ames testing protocol states that the initial culture should be around $1-2 \times 10^9$ cells/ml (Maron and Ames, 1983). Following the 12 hour growth period, all of the cultures tested had at least 10^9 cells/ml. The results reported here are expressed with their standard deviation, which is an uncommon practice in reporting Ames test results.

4.2.1 The effects of cloned *mucA/B*

DNA damaging agents that inhibit DNA replication induce the SOS response in *E. coli*. The SOS response is extremely rapid and induces over 30-40 proteins that help the cells overcome and deal with this damage (reviewed in Walker, 1984). Depending on the type of DNA lesion, *E. coli* may use a damage avoidance pathway where the polymerase switches strands to the non-damaged DNA template (Tang *et al.* 1998). However, situations occur where the only option for the cell is to replicate through the lesion, a process called translesion synthesis. Translesion synthesis gives the cells a chance to survive, but often has mutagenic consequences. Two key proteins which are involved in translesion synthesis are UmuC and UmuD. It has been known for some time that UmuC and UmuD play a role in translesion synthesis, however their exact role has only recently been elucidated. Tang *et al.* (1999) demonstrated in a reconstituted SOS mutagenesis assay that the mutagenically active UmuD'₂C complex, in combination with RecA, β sliding clamp, γ clamp loading complex, and SSB were able to bypass an abasic template lesion in a highly error prone manner in the absence of DNA polymerase III and

DNA polymerase II. They demonstrated that lesion bypass often resulted in preferential insertion of dATP opposite the lesion; an outcome that has been widely observed both *in vivo* and *in vitro* (the “A rule” (Strauss, 1991). Moreover, when a mutant UmuD’₂C104 was used, no detectable lesion bypass activity was observed. Taken together, the results suggest that UmuD’₂C is an “error prone” *E. coli* DNA polymerase (pol V) (Tang *et al.*, 1999).

Although UmuD’₂C is the first bona fide prokaryotic “error-prone” DNA polymerase to be described, it is likely that other related proteins possess the ability to catalyze phosphodiester bond formation. In fact, DinB protein (another SOS induced protein) has been recently shown to have DNA polymerase activity (Wagner *et al.*, 1999), and has been termed DNA polymerase IV. Unlike UmuD/C, the ability of MucA/B to act as a replicational accessory protein at sites of DNA damage has not yet been shown to result from DNA polymerase activity. However, due to the relatedness of these proteins and their similar effects in SOS mutagenesis, it is highly probable that this question will be addressed in the near future.

The importance of UmuD/C and MucA/B in SOS mutagenesis is clearly illustrated in the UV rifampicin assay (Figs. 16 and 17). The plasmid borne *mucA/B* genes present in an UmuC deficient host had a dramatic effect on the survival of the cells. In this assay, many cells that are expressing *mucA/B* do survive the UV treatment. In order to continue growth on rifampicin media, a mutation to the β subunit of RNA polymerase must have occurred as a result of SOS processing of the UV lesion. Rifampicin is a known transcriptional inhibitor that binds to the β subunit of RNA polymerase and prevents transcription (Nene and Glass, 1982). The effects of no

UmuD/C and MucA/B are clearly illustrated in the negative control (UmuC⁻ cells). The UmuC deficient cells cannot bypass the UV lesions and do not yield rifampicin resistant colonies following UV treatment (Figs. 16 and 17).

MucA/B had a noticeable effect on the Ames test results, particularly in the base substitution strains. When a pairwise comparison (see Results section 3.5.4) was made between two base substitution strains that differed only in the presence of MucA/B a large stimulation of base substitution mutations was observed for 2-NF, 1-NP, 1,6-DNP, NF, 4-NQO, and ICR-191 (Table 18). The low level of base substitutions in strains CBT407 and CBA407 is probably a direct result of the cell's inability to bypass DNA lesions; it is likely that the cells simply die (Tables 15 and 16). Since *S. typhimurium* LT2 contains *umuD/C_{ST}* and *samAB*, it is likely that several gene products play a role in base substitution mutagenesis (Nohmi *et al.*, 1991). However, the extent to which the different proteins promote error-free versus error prone bypass of lesions may be strongly influenced by gene dosage effects, and therefore the level of expression of *mucA/B* within the host cell is extremely important (Gruz *et al.*, 1998). For example, it has been shown that *mucA/B* is more efficient at enhancing base substitutions than endogenous UmuD/C and UmuD/C-like proteins (Lambert *et al.*, 1992; Shiba *et al.*, 1990; Urios *et al.*, 1993).

The frameshift locus *hisD3052* is a known hotspot for -2 frameshifts. Since the *hisD3052* target locus is a repetitive (CG)₄ sequence, -2 frameshifts are believed to occur by a slippage mechanism at this locus. The requirement for replicational accessory proteins such as MucA/B to promote mutagenesis in the frameshift strains is much less evident than is the case with the base substitution strains (Table 18). For example, when a pairwise comparison was done between the CF511 and CF507 frameshift strains (see

Result section 3.5.2), a marked increase in frameshift mutagenesis was observed for most chemicals, consistent with the notion that MucA/B plays a role in promoting frameshift mutagenesis (Table 18). However, the observed effect of MucA/B in augmenting frameshift mutation was relatively low when compared to the base substitution strains (Table 19).

The ability of replicational accessory proteins to promote mutagenic bypass of lesions is dependent on the chemical and whether the DNA adduct formed following nitroreduction blocks DNA polymerase III. For example *N*-(deoxyguanosin-8-yl)-1-aminopyrene, the major DNA adduct of 1-NP is a strong replication block and most likely requires the recruitment of replicational accessory proteins or alternative DNA polymerases to bypass the lesion (Vyas *et al.*, 1995; Gruz *et al.*, 1998). Conversely the major DNA adduct formed from 1,8-DNP, *N*-(2'-deoxyguanosin-8-yl)-1-amino-8-nitropyrene has been shown to not critically inhibit DNA replication at CG repetitive sequences (Gruz *et al.*, 1998; Lambert *et al.*, 1992). Moreover, 1,8-DNP still exhibited mutagenicity at the *hisD3052* even in the absence of replicational accessory proteins (Gruz *et al.*, 1998). In the current study 1,6-DNP showed significant mutagenicity in CFA507 (p213AB) and CFT507 (pCCOAB) in the absence of plasmid borne *mucA/B* although endogenous Umu-like proteins were still present (Tables 12 and 13). The *umuD/C_{ST}* operon has been shown to enhance -2 frameshifts at the *hisD3052* locus (Nohmi *et al.*, 1995). The *samAB* operon has also been shown to promote -2 frameshifts at the *hisD3052* locus to levels equal and even higher than MucA/B; however, this occurred only when a plasmid containing SamA'B was introduced into *S. typhimurium* YG5147 (Gruz *et al.*, 1998). The mechanism through which MucA/B, UmuD/C_{ST}, and

SamAB promote mutagenesis at the *hisD3052* locus, and the interplay between these DNA polymerases/replicational accessory factors has not yet been elucidated.

4.2.2 The Effects of OAT

Since OAT is involved in the metabolic activation of nitrosubstituted compounds it is not surprising that OAT increased the mutagenicity for strains containing this enzyme. Following nitroreduction the *N*-hydroxyarylamine species is further acetylated by OAT to form a transient intermediate which is unstable; cleavage of the N-O bond creates nitrenium ions. In the absence of OAT the *N*-hydroxyarylamine intermediate can spontaneously hydrolyze to form nitrenium ions, however it is believed that the presence of OAT accelerates the formation of this ion species (See Figs. 5 and 7).

The plasmids pYG213 and pYG219, which contain OAT, were constructed by Watanabe *et al.* (1990). OAT enzyme assays were carried out on both plasmids and pYG213 was found to exhibit approximately a 4-fold higher OAT activity than pYG219 (Watanabe *et al.*, 1990). In the present study, the ampicillin constructs and tetracycline constructs, derived from pYG213 and pYG219, respectively, did show an increase in mutagenicity in tester strains harbouring these plasmids. In strains containing OAT but no exogenous nitroreduction, i.e. CFA508 and YG1024, 1,6-DNP yielded over 4 and 5 million revertants/nmole, respectively (Tables 11 and 12). It has been shown previously with TA98/1,8-DNP₆, an OAT deficient tester strain, that maximal mutagenicity of 1,6-DNP and 1,8-DNP is dependent on OAT (McCoy *et al.*, 1983). Strains deficient in OAT are insensitive to the mutagenic action of several nitrosubstituted compounds including the dinitropyrenes (McCoy *et al.*, 1983).

4.2.3 The Effects of Nitroreduction

In terms of mutagenicity, the increased nitroreduction capacity of the strains constructed in this study resulted in an enhanced ability of both *hisG46* and *hisD3052* strains to detect mutations induced by several nitrosubstituted chemicals. It is difficult to single out one strain as the most sensitive frameshift or base substitution strain because there is some fluctuation in the results. However, among the new tester strains, those containing pCCOMA, p213MA, pCCOMBA, and p213MBA were extremely sensitive toward nitrosubstituted mutagens (Tables 12 and 13). When a pairwise comparison was made to illustrate the effect of NfsA in the matched frameshift strains CF511:510 (see Results section 3.5.2), only 2-NF, 1,6-DNP, and 2-NN showed a significant increase in mutagenicity (Table 18). The identical trend was observed for the base substitution strains (Table 19). However, when a pairwise comparison was made with both frameshift and base substitution strains containing plasmid-encoded NfsB, only 1-NP showed an increase in mutagenicity (Tables 18 and 19). When the mutagenic effect of both NfsA and NfsB was examined by a pairwise comparison of the frameshift strains CF511:CF508 (see Results section 3.5.2) only 2-NF, 1-NP, 1,6-DNP, and 2-NN showed an increase in mutagenicity (Table 18). The same type of comparison with the base substitution strains CB410:CB408 showed an increase in mutagenicity for chemicals 2-NF, 1,6-DNP, NF, and 2-NN (Table 19). It appears from the data that NfsA preferentially stimulates 2-NF, 1,6-DNP, 2-NN mutagenesis, while NfsB only stimulates 1-NP mutagenesis. In fact, NfsB may have an inhibitory effect on the mutagenicity of some chemicals, especially 1,6-DNP. The fact that NfsB stimulates 1-NP mutagenesis is supported by published mutagenicity test results for YG1021 and YG1041 (Watanabe *et*

al., 1990; Hagiwara *et al.*, 1993). These strains contain the *cnr* gene i.e. the *nfsB* homolog in *S. typhimurium*.

The strong observed effects of OAT in enhancing the mutagenicity of 1,6-DNP in frameshift strains containing pCCOM (for example, CFT508) or p213M (for example, CFA508) have implications regarding the nature of the nitroreductase species involved in reduction of this potent mutagen to a proximate mutagenic species. In these strains, there is no plasmid encoded nitroreductase activity and the number of revertants/nmole induced by 1,6-DNP is extremely high, ranging from approximately 500,000 to almost 5 million revertants per nanomole (Tables 12-13 and 15-16). OAT acts on the *N*-hydroxyarylamine intermediate which results from nitroreduction in the metabolic pathway; the *hisD3052* derivatives used in these studies are deficient in *SnrA*, and, as described above, our results show that the classical nitroreductase *Cnr* (*NfsB*) present in *hisD3052* strains is inhibitory with respect to metabolic activation of 1,6-DNP (Table 18). It has been shown that 1,6-DNP and 1,8-DNP still exert their mutagenicity in TA98NR; a *cnr* and *snrA* deficient strain (Rosenkranz and Mermelstein, 1983; Bouramandi *et al.*, in preparation). All of these results suggest that there must be another yet unidentified nitroreductase acting on 1,6-DNP. One possibility is that the dinitropyrenes may be substrates for the oxygen sensitive type II nitroreductase(s). However, the results presented here do show an increase in mutagenicity when *nfsA* is present in the strain, suggesting that *NfsA*, if present, can act on 1,6-DNP.

The high nitroreductase activity of these strains does not necessarily guarantee an equivalent increase in mutagenicity. It is not known which step in the nitroreductive pathway that the NFR assay is monitoring. For example, it is unclear whether the

reduction at 400 nm corresponds to the $\text{NO}_2 \rightarrow \text{N=O}$ step or subsequent steps in the pathway that ultimately give rise to the biologically inactive NH_2 species (see Fig. 5). The degree to which the mutagenicity has increased in the new strains is dependent on the type of mutation the chemical preferentially induces and whether the DNA adduct produced following nitroreduction efficiently induces that type of mutation. Since no attempt was made to identify and quantify the DNA adducts produced following nitroreduction for the nitrosubstituted chemicals used in this study, it is difficult to accurately assess the differences in mutagenicity. There may be additional factors such as the preferred substrate of NfsA versus NfsB. It is unclear what step NfsA or NfsB catalyze in the overall reduction of the NO_2 group, or whether there is any preference of the two enzymes for different substrates. For example, NfsA may efficiently catalyze the first step i.e. $\text{NO}_2 \rightarrow \text{N=O}$, but be less efficient at the remaining nitroreduction steps whereas NfsB may catalyze the $\text{N=O} \rightarrow \text{NHOH}$ step well, but be inefficient in the other nitroreduction steps (see Fig. 5).

4.3 Comparison of the new frameshift tester strains to the previously established strain YG1041

In order to compare the mutagenicity test results of the previously established tester strains with the new strains developed in this study, it was important to test the former strains under the same experimental conditions. Even though the assay is simple and quick, there are various aspects of the protocol that can introduce significant interlaboratory variation (Friederich *et al.*, 1982). Much effort was taken to minimize sources of variation by pre-aliquoting solutions, the use of a plate pourer, and a pre-

defined growth time. However, it is impossible to eliminate every source of variation. The use of an automatic colony counter is essential in this type of work, but it can also be a large source of variation if it is not calibrated properly (Claxton *et al.*, 1984).

A pairwise comparison was made between the frameshift strain CFT511 and the base substitution strain CBT411 and strains CFA410:CBA410 to see which chemicals preferentially induced frameshift mutations (Table 17). It is clear from this data, that 2-NF, 1-NP, and 1,6-DNP preferentially induce frameshift mutations rather than base substitution mutations. Similarly, the same comparisons were made with the previously established strains YG1041:YG1042 and the identical trend was observed (Table 17).

The observed number of revertants/nanomole for most of the previously established tester strains was usually in the range of the published values. For example, TA98 treated with 2-NF yielded 50 revertants/nmole (Table 11) whereas the published values ranges from 18-88 revertants/nmole (Hagiwara *et al.*, 1993; Rosenkranz and Mermelstein, 1983). There is one exception where the observed values for 2-NF are drastically different from the published values. YG1041 treated with 2-NF yielded 300 rev/nmole and the published value was reported to be 52, 000 rev/nmole for this strain (Table 20) (Hagiwara *et al.*, 1993). Due to this extreme discrepancy in the number of rev/nmole for this compounds, a new 2-NF stock was purchased. Following testing with the new stock, the number of revertants/nmole was still out of range of the published value. As for 1-NP, two strains, CFA509 (p213MA) and CFT509 (pCCOMA) yielded more revertants/nmole than YG1041 and the published value for YG1041 (Table 20). No new strains yielded a higher number of revertants/nmole for 1,6-DNP than YG1041 (Table 20). Among the test strains developed in this study, the mutagenicity of 1,6-DNP

Table 20. Comparison of the new *S. typhimurium* frameshift tester strains to the previously established strain YG1041.

Bold type indicates a result that is higher than the published value for that chemical.

Blue type indicates a tetracycline resistant strain.

Red type indicates an ampicillin resistant strain.

* Value reported is for 1,8-DNP.

Chemical	Strain	Induced His ⁺ revertants/nmole	Induced His ⁺ revertants/nmole for YG1041 (<i>mucA/B</i> , <i>OAT</i> , <i>cnr</i>)
2-NF	YG1041	320	52,383
	CFA509	1,900	
	CFA511	1,720	
	CFT509	1,930	
	CFT511	3,570	
1-NP	YG1041	130,000	161,461
	CFA509	175,000	
	CFA511	146,000	
	CFT509	227,000	
	CFT511	50,200	
1,6-DNP	YG1041	8,410,000	8,436,283*
	CFA509	6,240,000	
	CFA511	2,250,000	
	CFT509	2,620,000	
	CFT511	6,640,000	

was highest (more than 6 million revertants per nanomole) in CFT511 and CFA509. This can be compared to approximately 50,000 revertants per nanomole in the conventional Ames strain, TA98.

4.4 Comparison of the new base substitution tester strains to the previously established strain YG1042

The number of revertants/nmole reported for most of the new base substitution strains was significantly greater than values previously reported for the battery of nitrosubstituted chemicals, except for 2-NN (Table 21). However, the base substitution strain CBT411 yielded more revertants/nmole with 2-NN than YG1042 within this study (Table 21). With 4-NQO the four strains possessing pCCOMA, p213MA, pCCOMBA, and p213MBA all showed a significant increase in the number of revertants/nmole when compared to YG1042 (Table 21). The identical trend was observed with NF as the same four strains yielded higher revertants/nmole than YG1042 (Table 21). The increased nitroreduction in these strains is likely responsible for the increased mutagenicity.

Table 21. Comparison of the new *S. typhimurium* base substitution tester strains to the previously established strain YG1042.

Bold type indicates a result that is higher than the published value for that chemical.

Blue type indicates a tetracycline resistant strain.

Red type indicates an ampicillin resistant strain.

Chemical	Strain	Induced His ⁺ revertants/nmol	Induced His ⁺ revertants/nmole for YG1042 (<i>mucA/B</i> , <i>OAT</i> , <i>cnr</i>)
2-NN	YG1042	120	260
	CBA409	60	
	CBA411	30	
	CBT409	60	
	CBT411	170	
4-NQO	YG1042	1,480	1,192
	CBA409	8,690	
	CBA411	2,220	
	CBT409	3,900	
	CBT411	5,920	
NF	YG1042	260	154
	CBA409	390	
	CBA411	1,020	
	CBT409	260	
	CBT411	170	

5.0 CONCLUSIONS

The focus of this work was to genetically modify conventional Ames tester strains TA1535 and TA1538 by introducing plasmids which encode the major and minor *E. coli* nitroreductase genes in different combinations with *S. typhimurium* OAT and *E. coli* *mucA/B*. The following conclusions can be made from this work:

- JVQ3 cells containing plasmids encoding *nfsA* showed large killing zones. The largest killing zone was observed with cells harbouring pCCOAB. Comparison of strains lacking *nfsA* containing plasmids to strains harbouring *nfsA* containing plasmids suggested that the presence of NfsA had a significant effect on nitrofurazone toxicity.
- Plasmids p213M, p213MB, pCCOM, and pCCOMB containing cloned *mucA/B* showed an UV dose dependent increase in the frequency of formation of rifampicin resistant mutations indicating the presence of functional MucA/B proteins.
- Nitrofurazone reductase assays with strains harbouring nitroreductase genes and tetracycline resistance markers had much higher enzyme activities than their counterparts encoding ampicillin resistance markers. Strains containing *nfsA* constructs had higher enzyme activities when compared to *nfsB* constructs, and the highest enzyme activity came from strains containing pCCOMBA.
- CFA509, CFT509, CFT511, and CFA511 were the most sensitive frameshift strains. Pairwise comparisons between frameshift strains that differ with respect to *nfsA* revealed that NfsA preferentially stimulated frameshift mutations when 2-NF, 1,6-DNP, and 2-NN were used as test chemicals. The presence of NfsB did not stimulate

frameshift mutagenesis for most nitrosubstituted chemicals, the only exception being 1-NP.

- CBA409, CBA411, CBT409, and CBT411 were the most sensitive base substitution strains. Pairwise comparisons between strains that differ with respect to *nfsA* revealed that NfsA stimulated base substitution mutagenesis when cells were treated with 2-NF, 1,6-DNP, 2-NN, and, to a lesser extent, NF. The presence of NfsB only stimulated base substitution mutagenesis in cells treated with 1-NP.
- The effect of MucA/B on frameshift mutagenesis was evident in cells treated with 2-NF, 1-NP, 1,6-DNP, NF, and 2-NN.
- MucA/B significantly enhanced base substitution mutagenesis for most compounds, particularly 2-NF, 1-NP, 1,6-DNP, NF, 4-NQO, and ICR-191.
- The newly constructed frameshift and base substitution strains possessed a broader substrate specificity and, in some cases, were more sensitive toward nitrosubstituted chemicals than the previously established strains.

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