

Defining the mechanism of NAD-mediated mutagenesis

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Abstract

Bacteria require the ability to rapidly evolve and adapt in order to overcome the physiological stresses that they often face. Genetic variation and natural selection drive adaptive mutation, however the mechanism behind adaptive mutation is controversial. A novel discovery indicated adaptive mutation, through hypermutation, occurs in *Mesorhizobium loti*.

Mesorhizobium loti is an α -proteobacterium and is a nitrogen-fixing microsymbiont of several *Lotus* species. The investigations for this study were carried out using three *M. loti* strains: *M. loti* R7A, *M. loti* R7ANS and *M. loti* R7A Δ dct. *M. loti* R7A contains a mobile symbiosis island, ICEM/Sym^{R7A}, *M. loti* R7ANS is a non-symbiotic derivative of R7A that lacks ICEM/Sym^{R7A}, and *M. loti* R7A Δ dct contains a deletion of the otherwise-functional C4-dicarboxylate transport system that is encoded on ICEM/Sym^{R7A}. These strains contain a non-functional (cryptic) C4-dicarboxylate transport (*dct*) system on their chromosome and thus they are unable to grow on media with succinate as a sole carbon source. However when cultured on such media, a sub-set of the strains give rise to Dct⁺ colonies over time that contain mutations that activate the cryptic *dct* genes.

This ability to mutate was found to be due to *nadQABC* genes located on the mobile symbiosis island, ICEM/Sym^{R7A}. It was found that in the presence of the *nadQABC* locus, or of a large excess of nicotinic acid (NA), the Dct⁻ non-symbiotic mesorhizobia and strain R7A Δ dct give rise to Dct⁺ mutants over time. Strains lacking the *nadQABC* genes and grown in the presence of low concentrations of NA never give rise to Dct⁺ mutants. Furthermore the mutants seem to arise only after growth has stopped. Mutagenesis under non-growing conditions is known as stress-induced mutagenesis (SIM) or stationary-phase mutagenesis.

The purpose of this study was to investigate the hypothesis that the Dct⁺ mutants arise as a result of oxidative DNA damage. We propose that hypermutation should result in accumulation of mutations in the genome due to oxidative stress caused by an imbalance in NAD homeostasis and that potentially deleterious mutations will be repaired.

In this study, a series of single gene insertion duplication mutants (IDM) and a markerless deletion mutant were constructed for genes involved in DNA repair and in oxidative stress tolerance. It was hypothesised that these mutants would show an increase in both deleterious and beneficial mutations. The genes that were targeted encode proteins involved in the GO repair system (MutM, MutT, MutY) and Mismatch Repair (MMR) system (MutS) as well as a

monofunctional catalase (KatE) and a bifunctional catalase/peroxidase (KatG). Mutants in each of the genes were constructed in *M. loti* strains R7A, R7ANS and R7A Δ dct by either insertional duplication mutagenesis or in-frame deletion and mutants confirmed via PCR and Southern hybridisation techniques.

The mutants in the R7ANS and R7A Δ dct backgrounds were assayed for mutator phenotypes in the presence or absence of an excess of NA. The results demonstrated a considerable increase in mutation rate for the *katG*, *mutS*, *mutT* and *mutY* mutants, while the *katE* and *mutM* mutants showed wild-type mutation rates. Overall, these results provide compelling evidence for a mechanism whereby under conditions of stress, elevated levels of ROS and NADH lead to HO $\cdot$ formation via the Fenton reaction. This damage leads to wide-spread mutation resulting in both deleterious and beneficial mutations. The deleterious mutations are constantly repaired, and when a beneficial mutation occurs, the conditions of stress are alleviated, removing the source of SIM and restoring mutation rates to their original state.

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List of Abbreviations

[w/v]	weight to volume ratio	NA	nicotinic acid
°C	degrees Celsius	NAD	nicotinamide adenine dinucleotide
µg	microgram	NaNM	nicotinate mononucleotide
µm	micrometre	O ₂	molecular oxygen
ATP	Adenosine triphosphate	O ₂ ^{·-}	superoxide anion
BLAST	basic local alignment search tool	OD	optical density
bp	base pair	ORF	open reading frame
cfu	colony forming units	PCR	polymerase chain reaction
CG	cryptic growth	RDM	rhizobium defined media
cm	centimetre	Rif	rifampicin
Dct	dicarboxylate transport	ROS	reactive oxygen species
dH ₂ O	distilled H ₂ O	RPM	revolutions per minute
DM	directed mutation	s	second
DNA	deoxyribonucleic acid	SIM	stress induced mutagenesis
dNTP	deoxynucleotide triphosphate	SOD	superoxide dismutase
DSB	double-strand break	ssDNA	single-stranded DNA
DSBR	double-strand break repair	t-BHP	tert-butyl hydroperoxidase
g	gram	Tc	tetracycline
<i>g</i>	gravitational force	TLS	translesion-synthesis
GO	8-oxo-G	TY	tryptone yeast
GRDM	glucose RDM		
h	hour		
H ₂ O ₂	hydrogen peroxide		
HM	hypermutation		
HO [·]	hydroxyl radical		
IDM	insertional duplication		
	mutagenesis		
kb	kilobase		
LB	Luria-bertani		
mm	millimetre		
mM	millimolar		
MMR	mismatch repair		

1

Introduction

1 Introduction

Bacteria are ubiquitous organisms that are exposed to all aspects of the Earth's ever-changing conditions. Bacteria require the ability to rapidly evolve and adapt to match environmental flux in order to survive and reproduce. To overcome the physiological stresses that they can face, bacteria have evolved mechanisms to allow significant changes in metabolism as well as gene expression. Genetic variability is a vital component of an evolving organism, and it stems primarily from both recombination and mutagenesis (Bjedov, Tenaillon *et al.* 2003).

Evolution relies on two components: random heritable genome variation and natural selection. As new variation is created, the environment selects for those organisms most well suited to their ecological niche, however the mutations are thought to be independent of their phenotypic consequence (Luria and Delbrück 1943; Newcombe 1949). Luria and Delbrück, (1943) showed early on that mutations can arise randomly without selection as the cause, and for years it was believed that mutation occurs at a constant rate and at random throughout a genome. This idea advocates a model in which a particular stress on a population selects for previously established mutations existing in a subset of organisms, which subsequently gain advantage over the rest of the population. Although this is a valuable model, it is too simplistic to explain many of the observations that have been recorded over time.

Cairns *et al.*, (1988) suggested that there may be processes that could direct mutagenic change. Since then many different mechanisms have been identified that enable genetic variation to be produced when an organism is under stress (Foster 2007). Adaptive mutation is used to describe a concept where mutations occur in response to an environment in which the mutations were selected (Rosenberg 2001; Rosenberg 2001). There are currently several models that attempt to provide a mechanism for adaptive mutation: directed mutation (DM), hypermutation (HM), and cryptic growth (CG) (Rosenberg and Hastings 2004). These last two models are considered the likely candidates, yet there is much controversy regarding the mechanism that explains the phenomenon of adaptive mutation (Roth, Kugelberg *et al.* 2006). In particular, Roth *et al.* consider that any mechanism that increases mutation rate under stress would be evolutionarily disadvantageous because of the large excess of deleterious over beneficial mutations that it would produce.

The hypermutation model is synonymous with stress-induced mutagenesis (SIM) or stationary phase mutagenesis, a theory that involves induction of a mutagenic state in response to stressful conditions, e.g. nutrient limitation (Rosenberg and Hastings 2004). The cryptic growth

model is synonymous with amplification mutagenesis, and is based on the theory that “small-effect” mutations caused by recombination-mediated gene amplification are very common but are unstable. When a particular selection pressure is placed on a population, pre-existing cells with an amplification of genes that relieve the pressure, proliferate and out-compete the rest whilst providing a larger target for mutagenesis. Once a mutation occurs, the amplification is usually lost, giving the appearance of an increase in mutation rate (Roth and Andersson 2004).

Mesorhizobium loti is an α -proteobacterium and is a nitrogen-fixing microsymbiont of several *Lotus* species (Sullivan, Patrick *et al.* 1995). The investigations for this study are being carried out using three *M. loti* strains: *M. loti* R7A, *M. loti* R7ANS and *M. loti* R7A Δ dct. *M. loti* R7A is derivative of ICMP3153 containing a mobile symbiosis island, ICEM/Sym^{R7A} (Sullivan, Trzebiatowski *et al.* 2002). *M. loti* R7ANS is a non-symbiotic derivative of R7A that lacks ICEM/Sym^{R7A} (Ramsay, Sullivan *et al.* 2006). *M. loti* R7A Δ dct contains a deletion of the otherwise-functional C4-dicarboxylate transport system that is encoded on ICEM/Sym^{R7A} (Weaver 2003). It was found that non-symbiotic mesorhizobia are auxotrophic for biotin, thiamin and most were auxotrophic for nicotinamide (Sullivan, Eardly *et al.* 1996).

These strains contain a non-functional (cryptic) C4-dicarboxylate transport (*dct*) system on their chromosome and thus they are unable to grow on media with succinate as a sole carbon source (Weaver 2003). However when cultured on such media, a sub-set of the strains give rise to Dct⁺ colonies over time that contain mutations that activate the cryptic *dct* genes. This has been tested by streaking for single colonies onto succinate media, which in most cases, resulted in the expected Dct⁻ phenotype. Eight non-symbiotic mesorhizobial strains were tested (CJ1-CJ7 and N18) and they gave rise to three distinct phenotypes (Figure 1.1). CJ2, CJ3, CJ4 and CJ6 showed a definitive succinate negative phenotype over a prolonged incubation, they only formed microcolonies through utilisation of trace carbon sources present in the media, but no Dct⁺ colonies arose (Figure 1.1A). Strains CJ5 and CJ7 showed the opposite result, and grew successfully on succinate (Figure 1.1C). In two of these strains (CJ1 & N18), however, it was observed that, although initially unable to utilise succinate, some Dct⁺ colonies arose over an extended incubation (Figure 1.1B). Strain R7A Δ dct also initially formed microcolonies on media with succinate as sole carbon source but gave rise to a high frequency of Dct⁺ mutant colonies over a period of time (Ashbrook 2012; Sullivan Unpublished data).

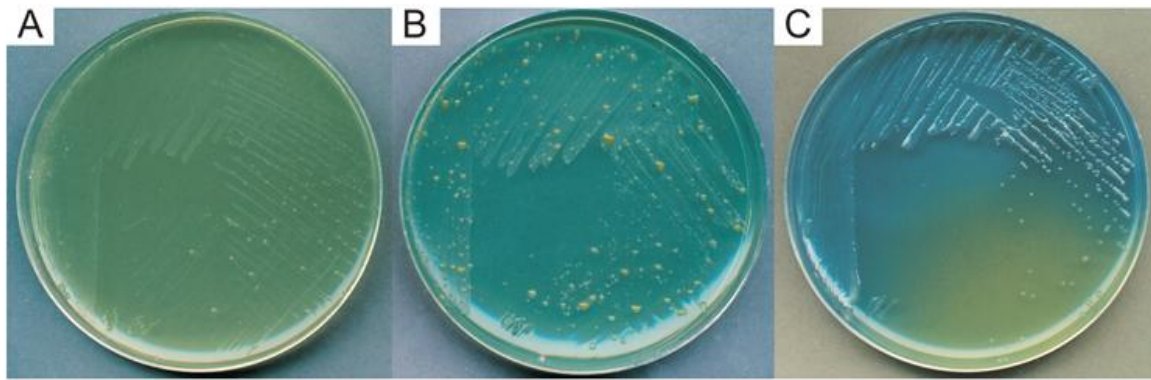


Figure 1.1 *M. loti* succinate growth phenotypes reproduced from (Weaver 2003). Images of non-symbiotic mesorhizobial strains that show 3 distinct growth phenotypes on media containing succinate as the sole carbon source. (A) Dct⁻ phenotype (CJ2, CJ3, CJ4 CJ6); (B) Dct⁻ → Dct⁺ (CJ1 and N18); (C) Dct⁺ (CJ5 and CJ7).

To confirm that the observed Dct⁻ phenotype was due to a lack of the functional *dct* locus, a recombinant plasmid containing *dctABD* genes from *Rhizobium leguminosarum* (Ronson, Astwood *et al.* 1984) was introduced into strains unable to utilise succinate. The addition of this plasmid converted these strains to the Dct⁺ phenotype, which confirmed that it is the *dct* locus that is required for successful succinate utilisation.

This ability to mutate was found to be due to *nadQABC* genes present on a plasmid found in strains CJ1 and N18 and on the symbiosis island in strain R7AΔ*dct* (Ashbrook 2012; Sullivan Unpublished data). It was found that in the presence of these *nadQABC* genes, or in the presence of a large excess of NA (50 µg/ml), the Dct⁻ non-symbiotic mesorhizobia and strain R7AΔ*dct* give rise to Dct⁺ mutants over time, whereas these strains lacking the *nadQABC* genes and grown in the presence of concentrations of NA less than 25 µl/ml never give rise to Dct⁺ mutants. Furthermore the mutants seem to arise only after growth has stopped i.e. the mutations occur in non-growing or very-slow-growing cells (Sullivan Unpublished data). Mutagenesis under non-growing conditions has been called stress-induced mutagenesis (SIM, the term used in this thesis) or stationary-phase mutagenesis.

It is known that NADH is toxic during oxidative stress and contributes electrons for iron reduction resulting in formation of hydroxyl radicals from hydrogen peroxide (H₂O₂). However, phosphorylated NADH (NADPH) is protective and provides electrons for repair and deoxynucleotide synthesis (Grose, Joss *et al.* 2006). It seems plausible that strains grown in an excess of NA may have their NADH/NADPH homeostasis upset leading to oxidative stress and resultant DNA damage that could be causing the observed increased mutation rate.

The purpose of this study was to investigate the hypothesis that the Dct⁺ mutants arise as a result of oxidative DNA damage. To investigate whether a genome-wide increase in mutation rate is occurring under these conditions, a series of single-gene knock-out mutants were constructed that are missing components of their oxidative stress response or DNA repair systems. The genes that were targeted encode proteins involved in the GO repair system and Mismatch Repair (MMR) system as well as a monofunctional catalase and a bifunctional catalase/oxidase.

The remainder of this Introduction covers the various oxidative stress response and repair systems that were targeted for mutagenesis as well as Stress Induced Mutagenesis (SIM), and oxidative stress. The oxidative stress response and DNA repair systems are detailed, showing how these systems function, the roles of the various proteins involved, and the effect of oxidative stress. SIM is then reviewed in several systems to provide insight into the importance of SIM in adaptation and survival, as well as some of the previously elucidated mechanisms employed, and how processes like oxidative stress can drive SIM under conditions of stress. Finally, NAD metabolism and its relation to oxidative stress is explained in order to enlighten the reader of the mechanism behind the hypothesis of this study; NADH/NADPH imbalances under conditions of starvation lead to oxidative stress inside the cell and this drives an NAD-mediated SIM.

1.1 C4-dicarboxylate transport (Dct) system

Rhizobia are nitrogen-fixing symbionts of legumes and for this relationship to be effective, the bacteroids require a carbon/energy source which is supplied by the host plant to support growth and nitrogen fixation (Yurgel and Kahn 2004). The source supplied by the host are the C4-dicarboxylates malate, fumarate and succinate (Ronson, Lyttleton *et al.* 1981). This requirement of nitrogen fixation on the transport and catabolism of dicarboxylates has led to a large amount of research into the rhizobial dicarboxylate transport (Dct) system which is essential for nitrogen fixation (Ronson, Lyttleton *et al.* 1981);(Reid, Walshaw *et al.* 1996).

In 1984, Ronson *et al* investigated and cloned the Dct system genes. They discovered three *dct* genes, *dctA*, *dctB* and *dctD*. In a subsequent paper in 1987, Ronson *et al* were the first to presumptively identify a two-component regulatory system that responds to environmental stimuli, encoded by *dctB* and *dctD*. *dctB* encodes a sensor kinase which is thought to be activated by the binding of a C4-dicarboxylate to the periplasmic face of *dctB*. Activated DctB is an autokinase that then interacts with the N-terminal domain of DctD, resulting in phosphorylation of DctD. Phosphorylated DctD binds upstream of *dctA* and catalyses the ATP-dependent transition of RNA polymerase complexed with the alternative sigma factor RpoN and bound at the *dctA* promoter from a closed complex to an open complex resulting in the transcription of *dctA*, which encodes a membrane bound C4-dicarboxylate transporter (Ronson, Astwood *et al.* 1984; Ronson, Nixon *et al.* 1987; Yurgel and Kahn 2004). A summary of the induction of *dctA* expression by DctB and DctD is shown in Figure 1.2 (Weaver 2003). *M. loti* strain R7A, *M. loti* R7ANS and *M. loti* R7A Δ *dct* all contain a cryptic copy of the *dct* locus comprising the *dctABD* genes within their genome. *M. loti* R7A contains an active copy of the *dct* locus encoded on the mobile symbiosis island, ICEM/Sym^{R7A}.

1.2 Cryptic genes

Cryptic genes are defined as phenotypically silent DNA sequences that are not normally expressed during the life cycle of an individual but are capable of activation as a rare event in a few members of a large population by mutation, recombination, insertion elements, or other genetic mechanisms (Hall, Yokoyama *et al.* 1983). The presence of the cryptic copy of the *dct* locus in the strains used for this study allows for selection of colonies that have undergone mutation and reverted to a Dct⁺ phenotype, which is useful for investigating mutator phenotypes. Several experiments were performed in this study looking for mutational gain-of-function events that lead to the ability to utilise succinate as a sole carbon source. Because of the presence of cryptic genes in *M. loti*, it was very easy to screen for Dct⁺ colonies on media

containing succinate. Others have shown that the inability of strains with the cryptic *dct* system to utilise dicarboxylates is a property of the *dctB* gene and that most Dct⁺ mutants have undergone point mutation in either the *dctB* or *dctD* gene (Weaver 2003; Sullivan Unpublished data). This event is referred to as “reversion” to succinate utilisation in this thesis.

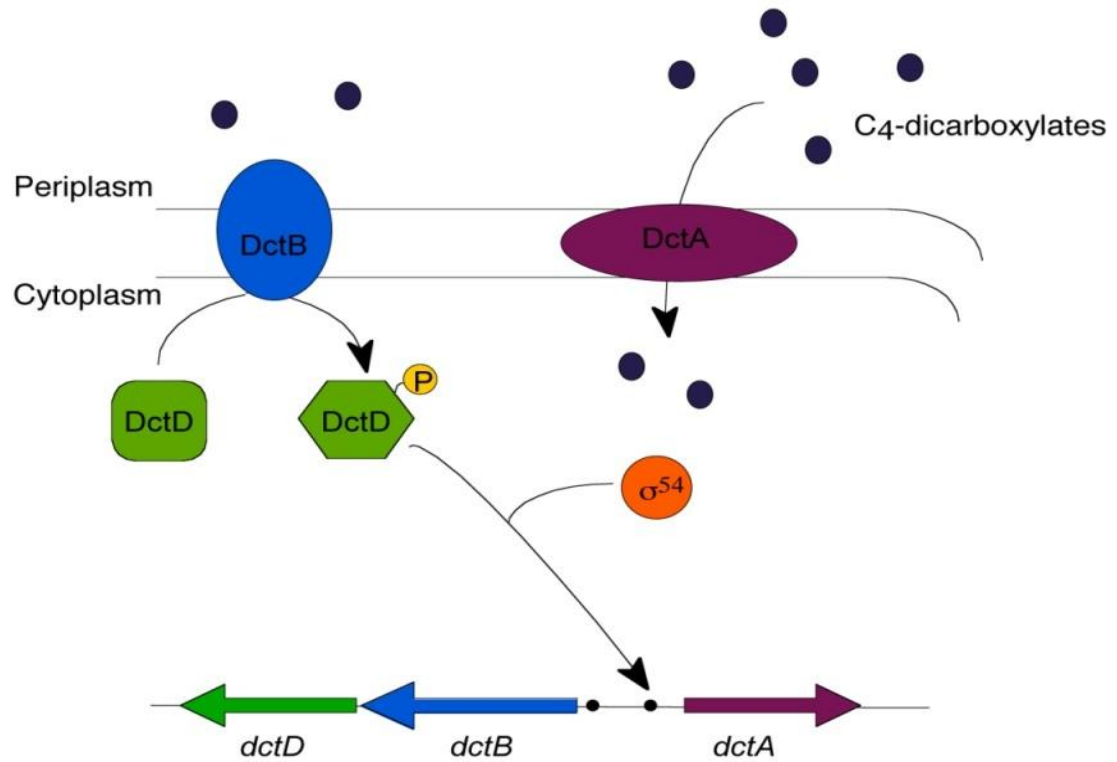


Figure 1.2 A schematic diagram of the Dct system. The *dctBD* genes encode a two-component regulatory system that activates the C4-dicarboxylate transporter, *dctA*. Reproduced from (Weaver 2003).

1.3 Adaptive mutation

“The origin of species by means of natural selection, or the preservation of favoured races in the struggle for life”, written by Charles Darwin (Darwin 1859), was a seminal paper in biology, giving rise to a simple and eloquent explanation of evolution and the means by which it occurs. It proposes that genetic variability occurs spontaneously and independently from the environment, which then subsequently selects those organisms most well-suited to survive. This idea was further supported in 1943, by Luria & Delbrück with their fluctuation test (Luria and Delbrück 1943) and for over forty years it remained dogma that spontaneous mutations arise as random errors during genomic replication (Foster 1999). It wasn’t until 1988 that this idea was challenged. A paper called “The Origin of Mutants” published by Cairns *et al* proposed that bacteria could choose or direct mutations in response to their environment (Cairns, Overbaugh *et al.* 1988) leading to the idea of directed mutation. However, this idea was discounted by showing that non-selected mutations also arose in the Lac⁻ population while the Lac⁺ mutations were occurring, ultimately giving rise to what is now known as the adaptive mutation phenomenon (Stumpf, Poteete *et al.* 2007). Adaptive mutation is the term used to describe these mutations formed in response to the environment in which they are selected (Rosenberg 2001). It is a process in which mutations occur in apparently static bacterial populations when subjected to non-lethal selective pressure (Foster 1999) and is synonymous with stress-induced mutation and stationary-phase mutation. The Cairns paper was important because it allowed for the selection of ‘soft’ or non-lethal mutations. Previous experiments, such as the Luria and Delbrück fluctuation test, utilised lethal selection and could not account for mutations that arose under selective conditions.

1.4 Luria-Delbrück and Cairns system experiments

The Luria-Delbrück experiment was carried out by exposing bacterial cultures to a virus in excess and then examining the resistant colonies after incubation. In their paper, they stated two main hypotheses that they wished to test, which they named the mutation hypothesis and the acquired hereditary immunity hypothesis (Luria and Delbrück 1943). They reasoned that if the mutation hypothesis was true, then the number of resistant colonies that appear would be highly variable between samples depending on the point during growth of the culture in which the spontaneous mutation occurred. If the acquired hereditary immunity hypothesis was correct then you would expect a relatively consistent number of resistant colonies to arise across samples. The random appearance of mutants including the odd “jackpot” culture, led to the formation of the Luria-Delbrück distribution. The problem with this experiment is that the

mutations had to arise before plating because it is a lethal selection; there was no possibility for mutations to arise in response to the selection pressure.

As mentioned earlier, in 1988 came the controversial paper that initiated the adaptive mutation debate, "The origin of mutants" (Cairns, Overbaugh *et al.* 1988) which introduced the idea of directed mutation. Three years later Foster and Cairns collaborated on a paper to further describe adaptive mutation in *Escherichia coli* (Cairns and Foster 1991). They acquired a strain, $\alpha 45$, that has a high reversion rate to Lac^+ after plating on minimal lactose medium. This strain contains a fusion of *lacI* and *lacZ* that is missing the *lac* regulatory region. Transcription initiates at the *lacI* promoter and is constitutive, however *lacI* contains a +1 frameshift mutation. This *lacI-lacZ* fusion is carried on an episome, F[']128 (Foster 2004). This F plasmid was mated into a *lac*⁻ *E. coli* strain that contained rifampicin resistance and was named FC40 (Foster 2004). When plated onto minimal lactose medium, Lac^+ colonies arose, initially showing a Luria-Delbrück distribution after 2 days, indicating these mutations had occurred pre-plating. However, Lac^+ revertants continued to arise over a week revealing a Poisson distribution, indicating that mutations were arising in the cells post-plating (Cairns and Foster 1991). These late arising -1 frameshift mutants only occurred on media containing lactose and in colonies that had appeared to have ceased growth. The late arising mutants would have occurred much sooner had they arisen pre-plating, since the revertants grow at wild-type rate.

1.5 Adaptive mutation controversy

This idea that mutagenesis could occur in response to environmental stimuli, as proposed by Cairns *et al.* (1988), led to extensive research and controversy about the mechanisms of mutagenesis employed by bacteria in order to adapt and evolve. Since then, many differing models have been proposed that seek to explain the observations derived from that system. The problem with the Cairns system is trying to determine whether selection is stimulating the formation of mutants, or simply allowing faster growth of pre-existing mutants that arise independently of selection. On one side is the adaptive mutation model, transiently increasing mutation rates to overcome the stress. The other model, known as amplification mutagenesis, argues in favour of a neo-Darwinist mechanism where the *lac* gene is amplified, with the amplification both allowing slow growth and increasing the number of copies of the gene so that the chance of a growth-dependent mutation is increased. Upon mutation the amplification is quickly resolved. Under this model there would be no need for an increased mutation rate.

1.6 Stress Induced mutagenesis (SIM) and the Cairns system

The transient hypermutation model is synonymous with adaptive, starvation-induced, stationary-phase and stress-induced mutagenesis; a theory that involves induction of a mutagenic state in response to stressful conditions (Rosenberg and Hastings 2004). This is the general model that has been proposed to explain the phenomenon of adaptive mutation observed in the Cairns system, as well as others. There is much debate, however, as to the mechanisms giving rise to stress-induced mutagenesis.

The first of these models is known as the recombination-dependent model and is proposed to stimulate mutation by inducing two stress response systems: the SOS response and the bacterial general stress response by transcriptional activator protein, RpoS.

When DNA damage occurs, areas of single-stranded (ss)DNA arise. This is caused by the damage itself or stalled replication forks, exposing ssDNA (Galhardo, Hastings *et al.* 2007). ssDNA is the SOS inducing signal and results in coating by RecA, the bacterial recombinase. In the Cairns system, however, it is not DNA damage leading to areas of ssDNA. In *E. coli* FC40, the *lacI-lacZ* fusion is carried on an episome, F[']128. Occasionally replication is initiated at *oriS* or *oriV*, the vegetative origin of the episome. Conjugation is not occurring but constant nicking at the conjugal origin, *oriT*, means that when a replication fork happens upon a nick at *oriT*, it becomes stalled leading to invasion of the ssDNA by RecA, resulting in a double-strand break (DSB). The DSB initiates the double-strand-break repair pathway (DSBR) which attempts to repair the DSB via homologous recombination. When DSBR is induced, a DNA polymerase, either Pol II or Pol IV, is recruited to prime DNA synthesis from the 3' end. If Pol IV predominates over Pol II then it may synthesise a track of error-containing DNA, which in the context of the *lac* region, will result in Lac⁺ mutations (Stumpf, Poteete *et al.* 2007).

RecA also plays another important role by de-repressing *lexA* expression, which results in upregulation of the error-prone polymerases. The ssDNA coated with RecA forms a nucleoprotein filament which can stimulate the autoproteolytic cleavage of LexA, a transcriptional repressor. Approximately 40 SOS genes are upregulated after the cleavage of LexA, including genes encoding proteins involved in DNA repair, recombination and DNA synthesis beyond lesions that block replication (Roth and Andersson 2004; Roth, Kugelberg *et al.* 2006). Three of the 43 SOS genes encode DNA polymerases: Pol II (*dinA*), Pol IV (*dinB*) and Pol V (*umuDC*) (Goodman 2002). Pol II is a B-family high-fidelity polymerase, whereas Pol IV

and Pol V are members of the Y-family of error-prone polymerases (Goodman 2002). After SOS induction, these 3 polymerases take the place of Pol III, the replicative polymerase and allow synthesis past blocking lesions on the template strand, which is known as translesion-synthesis (TLS) (Goodman 2002; Foster 2007; Galhardo, Hastings *et al.* 2007). Pol V is primarily involved in base substitution mutations targeted to chromosomal DNA damage sites in dividing cells (Schlacher and Goodman 2007), whereas Pol IV and, to an extent Pol II, are thought to be important in generating adaptive mutations in non-dividing cells. All 3 of these DNA polymerases confer a competitive fitness advantage in stationary-phase cell populations (Yeiser, Pepper *et al.* 2002; Schlacher and Goodman 2007).

In normally growing, undamaged cells, *dinB* is continuously expressed resulting in approximately 250 molecules of Pol IV (Kim, Matsui *et al.* 2001), a relatively high number for a polymerase. Under most conditions Pol IV has very little mutational effect (Foster 2007), however *E. coli dinB*, for example, is transcriptionally upregulated approximately 10-fold by the SOS response (Kenyon and Walker 1980; Kim, Matsui *et al.* 2001).

In addition to elevated levels of Pol IV induced by the SOS response, evidence has shown that there is an additional RpoS-controlled factor required for high levels of adaptive mutation (Layton and Foster 2003). Besides simply increasing the expression of *dinB* by two-fold (Layton and Foster 2003), RpoS, the general stress response regulator, provides an extremely important factor in the hypermutation model by limiting the mutagenic effects of Pol IV to a time of stress (Ponder, Fonville *et al.* 2005), allowing this mechanism of mutagenesis to be transient. Evidence has been provided that shows Pol IV is induced in stationary phase under the control of RpoS (Layton and Foster 2003). “At a bare minimum, the important role of RpoS in mutagenesis in this system indicates that mutagenesis is a stress response, regulated temporally by RpoS (and SOS)” (Galhardo, Hastings *et al.* 2007). The stress that most obviously and directly results in genetic change is exposure to DNA damaging agents (Foster 2007).

Another mechanism has been shown to exist in the FC40 system, the transient-hypermutation model, which is also known as hypermutation, general mutagenesis and general hypermutability (Stumpf, Poteete *et al.* 2007). Approximately 1% of Lac⁺ revertants that arise on lactose in the FC40 system have mutations throughout their genomes, therefore providing evidence of a state of hypermutation (Torkelson, Harris *et al.* 1997; Rosche and Foster 1999). Interestingly, when examined further, these revertants are found not to have increased mutation rates, indicating that the hypermutability must be transient. These genome-wide mutations have been shown to be both Pol IV- and SOS-dependent. Another interesting

discovery was the inactivation of the MMR system in the hypermutating subpopulation (Rosche and Foster 1999). As suggested by the authors, a plausible explanation to the lack of a functional MMR system in this hypermutating subpopulation would be a result of transcription and translation errors leading to defects in proteins that are involved in replication and repair (Rosche and Foster 1999). Furthermore, the spectrum of mutations occurring on the episome does not appear to differ between the hypermutating subpopulation and the non-hypermutating majority, therefore the simplest explanation is that the Lac⁺ generating mechanism does not differ across the population, however, the MMR-deficiency in the hypermutating subpopulation allows more of the Lac⁺ mutations to be retained (Rosche and Foster 1999).

In a general sense this model postulates that a subpopulation of cells, when placed under a particular stress, can modulate their mutation rate in order to hasten their ability to adapt and survive via an adaptive point mutation that alleviates the stress in question. It is a system where stress causes a transient condition of hypermutability which provides permanent mutations, some of them being beneficial (Rosenberg, Thulin *et al.* 1998). The problem with a state of increased mutability is the inevitable accumulation of deleterious and lethal mutations (Roth, Kofoid *et al.* 2003). It would be evolutionarily contradictory for an organism to enter a state that renders it less fit for survival, so for hypermutability to be plausible there must be some advantage conferred by a genome-wide increase in mutation rate. If an organism is placed on the brink of survival it becomes less difficult to imagine a situation where it would be evolutionary advantageous to undergo an increase in genome-wide mutagenesis. In a situation where mutation is the only chance for survival, it no longer matters if deleterious mutations arise because the outcome would be the same, however if the mutation that occurs is beneficial, then the organism will increase its chances of survival. Additionally, by reducing this genome-wide mutation rate to a small sub-population, further deleterious long-term effects are reduced.

1.7 Amplification mutagenesis and cryptic growth

Amplification mutagenesis, combined with cryptic growth, provides a model for adaptive mutation which is the predominant opposition to the theory of stress-induced mutagenesis. The amplification model has been proposed as an alternative theory to explain the appearance of *lac* revertants arising under selective conditions in the Cairns experiment (see above). This model proposes that stress simply acts to select a growing subpopulation of cells (10^6 of 10^8 plated cells) that contain a duplication of the *lac* gene (Slecht, Bunny *et al.* 2003). The *lac*

region is leaky providing 1-2 % of β -galactosidase activity compared with the control strain, which allows for slow-growing clones (Foster 1994). Selection favours the growth of the clones that contain *lac* amplifications. Growth of cells containing multiple *lac* alleles within a given clone increases mutational targets, therefore as *lac* targets are added to the clone the probability of a reversion event increases (Hendrickson, Slechts *et al.* 2002). When a large enough number of replicating *lac* alleles has been achieved, a reversion event occurs. Once reversion is achieved selection will favour any cells that retain the *lac*⁺ allele, and will counter-select those containing destabilising mutant alleles through segregation. The revertant cell overgrows the amplification clone and forms the final revertant colony that is observed (Roth and Andersson 2004). In this system, in theory, 10³ clones reach a population of 10⁵ cells each with up to 100 *lac* copies, therefore reaching 10¹⁰ replicating *lac* alleles. Cryptic growth argues against mutagenesis because this situation would allow for 100 revertants (observed number) at the normal mutation rate of 10⁻⁸/cell/division (Roth and Andersson 2004).

This model provides some explanations to the observations made by Cairns (1988) and Cairns & Foster (1991). However, this model cannot sufficiently explain all of the observations in the Cairns system. Evidence now shows that adaptive mutagenesis is a separate pathway to the point mutation model that cells can take for adaptive mutation. Although it can lead to mutations, it cannot account for the majority of mutants found in the Cairns system. It has been revealed that for the Cairns system, there are several mechanisms induced allowing cells to adapt and survive under stressful conditions. When looking at other species it is clear that there are a wide variety of mechanisms allowing for adaptive mutation, and over time it is likely that many more will be found.

1.8 Oxidative stress response systems

Oxygen is considered toxic to a cell through the generation of ROS. These reduced species are an inevitable outcome of an aerobic lifestyle, and research into oxidative stress has revealed numerous defence systems that offer protection from the damages that can occur when occupying aerobic habitats.

1.8.1 8-oxoguanine (GO) repair system

The 8-oxo-G (GO) repair system is initiated through oxidative stress and involves a series of DNA glycosylases and a pyrophosphohydrolase. When DNA undergoes damage, i.e. through reactive oxygen species (ROS), these glycosylases scan the DNA for the absence of duplex integrity (Imlay 2008). Hydroxyl radicals ($\text{HO}^\cdot$) possess the ability to attack any base, however, it is known that oxidative damage targets guanine in particular (Candeias and Steenken 1992). The biased targeting of guanine results in the formation of 8-oxo-7,8-dihydrodeoxyguanine (8-oxo-dG), an oxidized form of guanine that has the ability to pair with both cytosine and adenine, which in the case of adenine, results in G to T transversion events (Hogg, Wallace *et al.* 2005). There are three major enzymes involved in the GO system: MutM, MutY and MutT. MutM, also known as FPG (formamidopyrimidine DNA glycosylase) protein, is the main glycosylase that excises oxidized purines prior to replication (Tchou, Kasai *et al.* 1991). However, this process is not fool-proof and MutY is an adenine glycosylase that is responsible for removing G:A mispairs that may have occurred during replication, prior to excision by MutM (Imlay 2008). MutT is a nucleoside triphosphate pyrophosphohydrolase and is responsible for hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP, effectively reducing the amount of oxidised guanine available to DNA polymerase as a substrate for incorporation into DNA (Fowler and Schaaper 2006).

Studies on these proteins have revealed significant effects on mutation rates when their function is removed. *mutM* and *mutY* mutants in *E. coli* have both been shown to increase the number of G:C to T:A transversions (Cabrera, Nghiem *et al.* 1988; Nghiem, Cabrera *et al.* 1988; Michaels, Cruz *et al.* 1992) and further evidence revealed they work together, showing that a *mutM mutY* double mutant has a mutation rate 20-fold higher than the sum of the separate mutators (Michaels and Miller 1992). Strains deficient in *mutT* show a specific mutator phenotype. The *mutT* mutator phenotype relies on DNA replication and has a unidirectional mutational specificity limited to A:T to G:C transversions, with the strength of this mutator measured from 100- to 10,000-fold, depending on the marker tested (Yanofsky, Cox *et al.* 1966; Cox and Yanofsky 1967; Fowler and Schaaper 2006).

1.8.2 MMR system

DNA mismatch repair (methyl-directed mismatch repair) is a highly conserved system that helps maintain genome integrity through the correction of mismatched bases. Replication error is the main source of mismatches, however, biological processes can contribute significantly (Fukui 2010). *E. coli* MMR is the most well understood and involves many proteins. Initiation of MMR occurs through the function of three essential proteins: MutS, MutL and MutH. MutS functions as a homodimer. It is responsible for recognition and binding of mismatched DNA and is known as the mismatch recognition protein (Modrich and Lahue 1996). MutL also functions as a homodimer, and interacts physically with MutS. MutL helps to enhance mismatch recognition and also recruits/activates MutH (Li 2007). MutH is a methylation and sequence-specific endonuclease. Cleavage occurs 5' of a d(GATC) sequence on the unmethylated strand of hemimethylated duplex DNA (Ban and Yang 1998). The nick in the DNA that is caused by MutH results in the recruitment of DNA exonuclease, helicase, single-stranded DNA binding protein and polymerase which act to repair the missing sequence from the initial d(GATC) sequence beyond the mismatch (Modrich 1991).

1.8.3 Catalases

Hydrogen peroxide is a toxic compound that is converted via the Fenton reaction, to HO· which is the most potent ROS that causes DNA damage. Hydroperoxidases are enzymes responsible for catalyzing the decomposition of H₂O₂ to oxygen and water (Mongkolsuk, Loprasert *et al.* 1996). *E. coli* is known to encode two catalases; a monofunctional catalase and bifunctional catalase/peroxidase known as *katE* and *katG* respectively (von Ossowski, Mulvey *et al.* 1991). In *E. coli* *katE* and *katG* are under the control of separate regulators. *katG* expression is under the control of the transcriptional regulator OxyR and *katE* expression is under the control of stationary-phase sigma factor *rpoS* (Storz, Tartaglia *et al.* 1990; von Ossowski, Mulvey *et al.* 1991). Almost all organisms that live in aerobic conditions encode hydroperoxidases to avoid damage caused by ROS. *Mesorhizobium loti* MAFF303099 is a strain of *M. loti* that shows 98% chromosomal sequence identity with *M. loti* R7A. It has been shown experimentally that *M. loti* MAFF303099 encodes homologues of both *katE* and *katG* with the locus tags *mlr2101* and *mlr6940*, respectively (Hanyu, Fujimoto *et al.* 2009). It was found that a *M. loti* MAFF303099 *katG* mutant showed sensitivity to hydrogen peroxide under exponential growth and stationary-phase conditions. The *katE* mutant showed no response under exponential growth yet showed some catalase phenotypes during stationary-phase, leading the authors to

conclude that *katE* and *katG* are functionally separated, distinct catalases (Hanyu, Fujimoto *et al.* 2009).

1.9 SIM in other organisms

1.9.1 SIM in *Bacillus subtilis*

Section 1.2.3 detailed mechanisms of SIM shown to occur for the *lac* system in *E. coli* FC40. These are thought to be widespread mechanisms for adaptive mutation. Although *E. coli* has historically been the paradigm used to study adaptive mutagenesis, various other systems provide additional evidence for a variety of pathways that beget the same phenotypic observations of adaptive mutation, especially in the context of starvation, when looking at reversion from auxotrophy to prototrophy via mutation.

Extensive studies looking at the mechanisms underlying adaptive mutation have been carried out in various organisms but one of particular interest is *Bacillus subtilis*. Although *B. subtilis* is a gram-positive soil bacterium and *M. luti* is gram-negative, *B. subtilis* is relevant to this study as it has evolved in conditions much more similar to *M. luti* than *E. coli*. What is interesting about SIM in *B. subtilis* is that it seems to occur through almost completely different mechanisms than in *E. coli* FC40.

B. subtilis YB955 carries 3 amino-acid auxotrophies at the following alleles: *hisC952* (amber), *metB5* (ochre), and *leuC427* (missense) (Sung and Yasbin 2002). Stationary-phase mutagenesis has been demonstrated in this organism using an assay system for the reversion of these auxotrophic chromosomal alleles (Sung and Yasbin 2002; Robleto, Yasbin *et al.* 2007). This assay system has provided evidence demonstrating a significant difference in the underlying mechanisms of adaptive mutagenesis in *B. subtilis* and *E. coli*.

E. coli has been shown to require recombination processes and SOS induction in the generation of Lac⁺ SIM revertant cells, specifically functional RecA as detailed in section 1.6. This RecA requirement was investigated in *B. subtilis* strain YB9300 lacking a functional *recA* gene and was demonstrated to be unnecessary for stationary-phase mutagenesis (Sung and Yasbin 2002). Another surprising discovery was this system's lack of dependency for σ^B , the general stress sigma factor. In *E. coli* FC40, σ^S (RpoS) plays an influential role in the generation of adaptive mutations, and a lack of σ^S results in reduction of adaptive mutants and Pol IV (Robleto, Yasbin *et al.* 2007). However, in *B. subtilis* strain YB9102 (an isogenic derivative of

YB955 lacking a functional *sigB* gene) stationary-phase mutagenesis was unaffected (Sung and Yasbin 2002).

An interesting mechanism that was theorised after Cairns's paper was published in 1989 (Davis 1989) and has been revealed in *B. subtilis* is transcription-associated mutagenesis. Davis proposed the idea that the environment can stimulate selective transcription causing a bias on the randomness of mutation (Davis 1989), and thus offering a neo-Darwinian explanation to Cairns's Lamarckian idea of directed mutation. This mechanism of mutation through transcription is proposed to function through separation of the DNA strands caused by transcription, resulting in exposure of short regions of ssDNA that can potentially supercoil and form secondary stem-loop structures that expose vulnerable bases to mutagenic processes such as enzymatic, chemical or thermal attack (Davis 1989; Pybus, Pedraza-Reyes *et al.* 2010). This mechanism is consistent with some observations of SIM; it allows for DNA processing during stationary-phase along with a spatial specificity towards transcribed genes. Because the mutations are transcription associated, the chances of a widespread accumulation of mutations leading to a lethal genetic load would be reduced. Several studies in *B. subtilis* have provided evidence for the role of transcription in adaptive mutation.

Mfd (mutation frequency decline), also known as TRCF (transcription repair coupling factor), is an enzyme that is responsible for connecting transcription and DNA repair. Mfd recognises RNA polymerase that has stalled on a non-coding template site of DNA damage; it releases RNAP and its transcript as well as recruiting DNA excision repair machinery (Roberts and Park 2004). In *B. subtilis* it has been shown that inactivation of the Mfd protein results in a decrease in stationary-phase mutagenesis measured as the generation of reversions of the three alleles encoding amino acid auxotrophy (Ross, Pybus *et al.* 2006). There are three important features of Mfd-mediated stationary-phase mutagenesis: There is a significant reduction in revertants of the three alleles in the *mfd*-deficient strain, *mfd*⁺-mediated stationary-phase mutagenesis does not target tRNA genes, and the changes in the nonsense and missense alleles that led to prototrophy could be attained in the template strand by deamination, depurination, or oxidative damage (Ross, Pybus *et al.* 2006).

Along with the aforementioned components of mutagenesis in *B. subtilis*, it has also been suggested that there are hypermutable cell subpopulations. The hypothesis proposed that *B. subtilis* under periods of environmental stress could differentiate a hypermutable subpopulation that can randomly generate mutations to generate an increase in genetic diversity (Sung and Yasbin 2002). It was found the transcription factors ComA and ComK both

play a role in some aspect of stationary-phase mutagenesis in *B. subtilis*; it is believed that they are not directly involved in mutagenesis, but regulate the gene expression required for stationary-phase mutagenesis and also for differentiation of a hypermutable subpopulation. Moreover, it was suggested that a portion of these subpopulations become hypermutable by the suppression of DNA repair systems and/or activation of mechanisms able to damage DNA in the genome (Sung and Yasbin 2002). Following this suggestion, it was found that the MMR plays an important role in adaptive mutagenesis in *B. subtilis*.

MMR was originally linked to adaptive mutagenesis in the F' reversion system of *E. coli* (described above). The effects of MMR on stationary-phase mutagenesis have been analysed in *B. subtilis* with varying results from those found in *E. coli*. It was shown that a decrease in MMR in *B. subtilis* increases the number of revertants during stationary-phase (Pedraza-Reyes and Yasbin 2004). In a strain lacking a functional *mutSL* operon (MMR-deficient), the number of *his*⁺ revertants that accumulated was approximately 3 times higher than the parental strain. In the same MMR-deficient strain the number of *met*⁺ and *leu*⁺ revertant colonies that arose during stationary-phase were around 5 – 6 times higher than the parental strain (Pedraza-Reyes and Yasbin 2004). In addition to this, His⁺, Leu⁺, and Met⁺ revertant colonies were scored during stationary-phase for strains over-expressing the entire *mutSL* operon, or each of its components. The results with all three markers demonstrated a significant decrease in the number of revertant colonies that arose as a result of stationary-phase mutagenesis in the strains overexpressing the *mutSL* operon or the *mutS* gene. However, overexpression of *mutL* showed no significant effect (Pedraza-Reyes and Yasbin 2004). Overexpression of the *mutSL* operon was not sufficient in suppressing all of the stationary-phase mutants. This led the authors to suggest that they either did not generate enough MMR proteins, or there is some other mechanism involved in the generation of these mutants (Pedraza-Reyes and Yasbin 2004). The possibility that there could be other mechanisms involved compelled the authors to investigate the possibility that the GO repair system could be a component of adaptive mutagenesis in *B. subtilis* (Vidales, Cárdenas *et al.* 2009).

The GO repair system consists of 3 proteins: MutM, MutY, and MutT. The GO system (detailed in section 1.8.1) exists to prevent/reduce the impacts of oxidative damage, specifically 8-oxo-dGTP. 8-oxo-dGTP is incorporated into DNA opposite adenine, and MutT is responsible for hydrolysing this to a monophosphate version that blocks incorporation into DNA. When DNA is directly oxidised, 8-oxo-G is generated leading to transversion events. MutM and MutY are

DNA glycosylases that each function by removing 8-oxo-G from DNA when it has paired with cytosine or adenine respectively.

B. subtilis appears to encode a complete GO system; two putative 8-oxo-dGTPases (*mutT* & *ytkD*) (Kunst, Ogasawara *et al.* 1997), along with homologues of *E. coli* MutM and MutY, genes *mutM* and *ytkD* respectively (Sasaki, Yonemura *et al.* 2000). YtdK, an 8-oxo-dGTPase has been shown to hydrolyse 8-oxo-dGTP. It shares 9 of 10 absolutely conserved residues in the Nudix amino acid signature that has been shown in all proteins responsible for hydrolysing 8-oxo-dGTP (Ramírez, Castellanos-Juárez *et al.* 2004). Furthermore, it has been shown to complement the mutator phenotype of an *E. coli mutT* mutant (Ramírez, Castellanos-Juárez *et al.* 2004).

To test the effects of the GO system in *B. subtilis* on the production of stationary-phase induced mutagenesis, Vidales *et al.* constructed a series of mutants defective in GO system genes. They constructed single (*ytkD* & *mutT*), double (*ytkD mutT*) and triple knockout (*ytkD mutM yfhQ*) mutants. Both the *ytkD* and *mutT* deficient strains showed increased sensitivity to the oxidative stress agents H₂O₂ and *t*-BHP (tert-Butyl hydroperoxide), with the double mutant showing a more significant effect on the number of stationary-phase mutants. In the double mutant the number of His⁺ and Met⁺ revertants increased by three and four times compared with the parental strain, respectively. The triple mutant, a complete elimination of the GO system, had a dramatic effect on the number of His⁺ and Met⁺ revertants; with respect to the parental strain, there were 22 times more His⁺ and 50 times more Met⁺ revertant colonies during stationary-phase. Interestingly, in both the double and triple mutant, no significant difference was noted in the number of Leu⁺ revertants compared with the parental strain (Vidales, Cárdenas *et al.* 2009).

To assess the effects of the MMR system on these GO mutants, the *mutSL* operon was overexpressed in the triple mutant. Investigation into the spontaneous mutation rates to rifampicin resistance and reversion frequencies that occurred in growing cells in the GO-deficient strain, overexpressing the *mutSL* operon, revealed a dramatic decrease for the *his*, *met* and *leu* alleles. Similarly, in stationary phase, the tendency for the GO-deficient strain to accumulate His⁺ and Met⁺ colonies significantly decreased when the *mutSL* operon was overexpressed. Taking these results together leads to several convincing conclusions: oxidative stress provides an important source of DNA lesions during stationary-phase that contribute to the saturation of the MMR system; these mispairs can then be processed, concurrently, by the MMR and the GO system (Vidales, Cárdenas *et al.* 2009).

The work carried out on adaptive mutation in *B. subtilis* provides compelling evidence that oxidative stress plays a central role as a mechanism that generates genetic diversity promoting stationary-phase mutagenesis in starved cells of *B. subtilis*. The level of mutagenesis is reduced by both the MMR and GO repair systems.

1.9.2 SIM in *Pseudomonas*

The genus *Pseudomonas* is part of the *Gammaproteobacteria* class of bacteria. They are a ubiquitous group of organisms that inhabit a wide variety of ecological niches. Studies have been carried out in *Pseudomonas putida* to investigate the role of ROS in the generation of mutations for this organism under conditions of starvation, with the aim of elucidating mechanisms of stationary-phase mutagenesis in this system. Studies in *P. putida* have provided some important insights into the diversity of mechanisms employed for adaptation, some of which differ significantly from those in *E. coli* strain FC40.

A novel experimental system was developed in *P. putida* to assess the mutations that arise under conditions of starvation, along with the effect of growth phase on the rate of occurrence of these mutations (Kasak, Hůrak *et al.* 1997). The phenol degradation genes, *pheA* and *pheB*, confer the ability to use phenol as a carbon source. The promotorless *pheBA* operon was cloned into a plasmid (pEST1414) and introduced to *P. putida* strain PaW85. The *pheBA* gene cluster is used as a reporter to observe mutations that generate promoters for the transcription of *pheBA* genes. In starving *P. putida* cells, Phe⁺ mutants accumulate in the presence of phenol, but not without it. This study found that Phe⁺ mutants accumulate at differing rates, depending on the physiological state of the cell before plating. Phe⁺ mutants were found to accumulate at a higher rate in stationary-phase cells (Kasak, Hůrak *et al.* 1997).

Studies were carried out in *P. putida* providing evidence that different mutational pathways might be involved in early or late arising mutants in stationary-phase populations (Saumaa, Tover *et al.* 2002). The appearance of mutation type varied over time. At the beginning of starvation, C-to-A transversion was the dominant mutation type in the cell population, however, as starvation time increased, so did the proportion of 2- to 3-bp deletions. MutY is a glycosylase that is part of the GO repair system and is responsible for removing 8-oxo-G paired with adenine. In a *P. putida* MutY-deficient strain, the number of C-to-A and G-to-T transversion mutations increased in both growing and starving cells. The results obtained led the authors to suggest that the lower efficiency of the GO repair pathway in this organism could be the reason for the high frequency of C-to-A transversions at a particular site upstream of the *pheB* gene in early arising stationary-phase mutants. Investigation into the cause of the 2- to 3-bp deletions showed that it is a regulated process requiring the stationary-phase sigma factor RpoS, because these mutations were absent in an RpoS-deficient strain (Saumaa, Tover *et al.* 2002; Tarassova, Tegova *et al.* 2009). The authors conclude that the transversions represent mutations occurring at the beginning of starvation, when the DNA is likely to be less

protected. Over time the DNA becomes more resistant to 8-oxo-G mutagenesis, and the proportion of other types of mutation increase, which is likely to coincide with induction of a mutagenic DNA polymerase (Saumaa, Tover *et al.* 2002).

The role of Pol IV in mutagenesis of *P. putida* was investigated in a study by Tegova *et al* (Tegova, Tover *et al.* 2004). *P. putida* has a *dinB* gene and it was cloned into a plasmid under the control of the P_{lac} promoter. Overexpression of this *P. putida dinB* in growing *E. coli* led to a 24-fold increase in Rif-resistant mutants. The plasmid was then introduced into *P. putida* which resulted in an increase in the Rif-resistant mutants. Further experiments revealed 1-bp deletion mutants as well as the appearance of late arising Phe⁺ revertants rely on DinB function. After day 11 on selective media, the occurrence of revertant mutants was approximately 10-fold higher in wild-type *P. putida*, compared with the *dinB*-deficient strain (Tegova, Tover *et al.* 2004).

Interestingly in wild-type *P. putida*, when compared with a RecA-deficient strain, there was no difference in the accumulation of 1-bp deletions, indicating these *dinB* -dependent stationary-phase mutations do not require the RecA protein. The role of RpoS as a regulator of *dinB* was also investigated. The RpoS-deficient strain showed the same results as the wild-type indicating that the *dinB* promoter is not under the control of RpoS. Of note was the discovery that the transcription of *dinB* was about 3-fold higher in late stationary-phase compared to exponential phase (Tegova, Tover *et al.* 2004).

Further study investigated the direct role that ROS has on damage and defence systems in stationary-phase mutagenesis in *P. putida*. Saumaa *et al*, (2007) examined the effect of the GO system on the accumulation of stationary-phase mutants, specifically the effect of the GO repair enzymes MutM, MutY, and MutT, as well as Dps. Two assay systems were utilised, a chromosomal Rif system which allowed for detection of mutations in the *rpoB* gene and another system which detected base substitutions by eliminating the TAG stop codon in *pheA*, giving rise to Phe⁺ revertants. Under conditions of growth, the frequency of spontaneous Rif^R mutations and Phe⁺ mutations increased 90- and 80-fold respectively in a MutY-defective strain compared with the wild-type. In contrast, only a 2-to-3-fold elevation in mutation frequency was observed for MutM- and MutT-deficient strains. The effects of the GO repair enzymes during stationary-phase were then analysed using different assay systems to detect base substitution mutations and frameshift mutations separately. Under conditions of starvation, both the MutY- and MutT-deficient strains had an increase in base substitution mutations, resulting in an accumulation of Phe⁺ revertants, at an approximately 100- and 75-

fold rate respectively compared with the wild-type strain. However, under these conditions, the MutM-deficient strain showed no significant difference in the frequency of stationary-phase mutations compared with the wild-type (Saumaa, Tover *et al.* 2002). Dps is a stationary-phase specific DNA binding protein which protects cells from certain stresses in stationary-phase including oxidative stress. A *P. putida* Dps-deficient strain was constructed and compared with the wild-type under conditions of starvation. Dps appears to have no impact on the rate of Phe⁺ mutations accumulating in stationary-phase in *P. putida* (Saumaa, Tover *et al.* 2002).

Although RpoS showed no regulation of the *dinB* promoter (Tegova, Tover *et al.* 2004), it was shown to be required for 2- to 3-bp deletion mutations occurring in *P. putida* (Saumaa, Tover *et al.* 2002). In a 2009 study (Tarassova, Tegova *et al.* 2009), experiments were carried out to observe the role RpoS has in the occurrence of point mutations during stationary-phase in *P. putida*. An *rpoS*-deficient strain was compared with wild-type *P. putida* and showed a 1.5-fold increase in emergence of base substitution mutations during the first 4-6 days. Over a longer period (≥ 7 days), the number of Phe⁺ revertants in the *rpoS*-deficient strain was up to seven times higher than the wild-type. The general rate of the generation of base substitution mutations was then analysed using Rif resistance. The *rpoS*-deficient strain showed a 50-fold increase in Rif^R mutants compared to wild-type after 10 days of starvation (Tarassova, Tegova *et al.* 2009). Damage caused by ROS seemed the likely explanation for the increased mutation as well as decreased survival in the *rpoS*-deficient strain (Kivisaar 2010).

A series of mutants were constructed to assess the effect of oxidative damage in the strain lacking RpoS. One of the enzymes investigated was superoxide dismutase (SOD), the levels of which are approximately 5-fold lower in the *rpoS*-deficient *P. putida* strain. When SOD was overexpressed in the *rpoS*-deficient strain, a 10-fold increase in survival was recorded during long-term starvation. SOD also had an effect on the accumulation of Phe⁺ revertants, decreasing the frequency of accumulation of these mutants approximately 2.5-fold (Tarassova, Tegova *et al.* 2009). RpoS is known to positively regulate the expression of a catalase, KatB. A plasmid containing the catalase KatA, derived from *P. fluorescens* was introduced into the chromosome of *P. putida*. Expression of this catalase in RpoS-deficient *P. putida* showed a similar effect to the overexpression of SOD, increasing survival and reducing base substitution mutation frequency. Interestingly, overexpression of both SOD and the catalase simultaneously in the *rpoS*-deficient strain did not further improve survival and mutations rates indicating that SOD and catalase cannot fully prevent cell death under these conditions

(Tarassova, Tegova *et al.* 2009). The GO system mutants showed an increase in specific mutation type, however, the spectrum of mutations observed in the *rpoS*-deficient strain was a lot more varied. It appears that oxidative damage in this *rpoS*-deficient strain is not restricted to DNA damage, but is also targeting proteins and membrane lipids (Tarassova, Tegova *et al.* 2009; Kivisaar 2010).

In summary, it appears that in carbon-starved stationary-phase *rpoS*-deficient *P. putida*, oxidative stress elevates the mutation frequency due to DNA damage. At the same time the lack of RpoS results in a decline in repair fidelity as well as DNA replication through excessive oxidative damage of enzymes and/or errors during the translation of proteins (Tarassova, Tegova *et al.* 2009; Kivisaar 2010).

It is clear that there is no one mechanism responsible for generating SIM in bacteria; rather a multitude of factors play a role: oxidative damage of DNA and proteins, replication errors and DNA repair inefficiency. Bacteria are diverse in their genomic content, encoding different polymerases as well as repair machinery (Kivisaar 2010). Therefore no one species will reveal all there is to know about SIM. It is important for us to continue to explore many different systems to understand the various mechanisms employed to help our understanding of adaptation and evolution in bacteria.

1.10 NAD metabolism

Nicotinamide adenine dinucleotide (NAD) is a ubiquitous biological molecule that participates in many metabolic reactions (Lin and Guarente 2003). There are two basic pathways of NAD synthesis, a *de novo* biosynthetic pathway and a salvage biosynthetic pathway (Gazzaniga, Stebbins *et al.* 2009). The *de novo* synthesis pathway begins with the amino acid L-aspartate and eventually results in the synthesis of NAD after a series of enzymatic reactions (Figure 1.3). L-aspartate is converted to iminoaspartate by L-aspartate oxidase, encoded by *nadB*. *nadA* encodes quinolinate synthetase which, in conjunction with dihydroxyacetone phosphate, catalyses the condensation of iminoaspartate to quinolinate. *nadC* encodes quinolinate phosphoribosyltransferase which catalyses the reaction between quinolinate and 5-phosphoribosyl-1-pyrophosphate (PRPP) to give nicotinate mononucleotide (NaMN), pyrophosphate and CO₂ (Teramoto, Suda *et al.* 2010).

The salvage biosynthetic pathway permits the salvage of free bases, nicotinamide and NA (Liu, Foster *et al.* 1982). This pathway was defined by Preiss and Handler, (1958) who were working with human erythrocytes. The Preiss-Handler pathway is a three-step pathway that results in the formation of NAD from NA. The pathway then proceeds by consecutive formation of nicotinic acid mononucleotide and nicotinic acid-adenine dinucleotide, amidation then occurs resulting in the formation of nicotinamide adenine dinucleotide (Preiss and Handler 1958; Gazzaniga, Stebbins *et al.* 2009). The second and third steps of the Preiss-Handler pathway are common to both the NA *de novo* biosynthetic and salvage biosynthetic pathways because *de novo* biosynthesis produces NaMN (Gazzaniga, Stebbins *et al.* 2009).

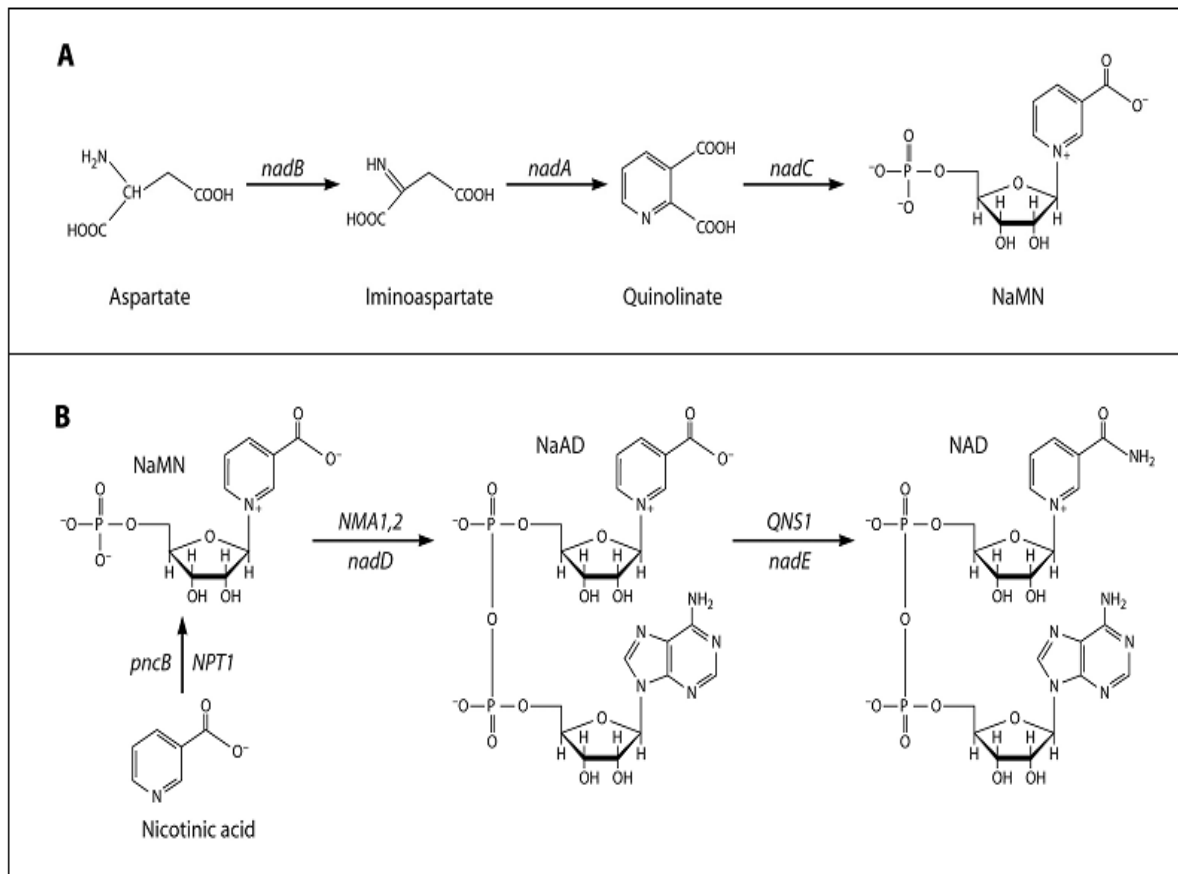


Figure 1.3 The two types of NAD synthesis pathways, reproduced from Gazzaniga *et al*, 2009: (A) *de novo* biosynthetic pathway encoded by the *nadABC* genes leading to NaMN. B) Salvage biosynthetic pathway, known as the Preiss-Handler pathway which is a three-step pathway that results in the formation of NAD from nicotinic acid. Steps 2 and 3 of the Preiss-Handler pathway (B) are common to both *de novo* biosynthetic and salvage biosynthetic pathways.

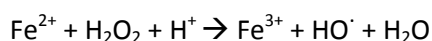
1.11 Oxidative stress and NAD

Oxidative stress is an inevitable outcome of the aerobic lifestyle. Molecular oxygen (O₂) is used by organisms to obtain energy through oxidation of nutrients and for respiration (Cabiscol, Piulats *et al.* 2000). An increase in the production of free radicals, induced by increased endogenous ROS generating systems or by exogenous oxidative insults, leads to an upset in the intracellular balance which is maintained by various antioxidants, resulting in a state of oxidative stress (Behl 1999).

Oxidative stress results from exposure to ROS such as hydrogen peroxide (H₂O₂), superoxide anion (O₂^{•-}), and hydroxyl radical (HO[•]), which are capable of damaging proteins, cell membranes and nucleic acids (Storz and Imlay 1999). Whenever molecular oxygen is responsible for chemical oxidation of electron carriers, H₂O₂ and O₂^{•-} are formed as a result (Storz and Imlay 1999). Molecular oxygen is a triplet species with a low univalent redox potential and is only able to accept one electron at a time, therefore molecular oxygen itself is unable to oxidise structural biomolecules (Woodmansee and Imlay 2002). However, oxygen can oxidise reduced flavins of redox enzymes resulting in the generation of these ROS.

NADH is an important co-enzyme used in oxidative phosphorylation and is a key component for respiration in cells. It has a crucial role as an electron donor transferring electrons to NAD dehydrogenase in the electron transport chain. Oxidative phosphorylation and the electron transport chain both function to drive the production of adenosine triphosphate (ATP), where oxygen acts as an electron acceptor. Cellular respiration can lead to the production of these ROS mentioned above (Derr, Faustoferri *et al.* 2012).

Cells normally maintain a tight NAD homeostasis, however under certain conditions this balance can be lost resulting in an imbalance of the ratio of NADH and NADPH. NADH is toxic during oxidative stress as it carries electrons from oxidative catabolic reactions and can contribute these electrons for iron reduction which results in the formation of HO[•] from H₂O₂ via the Fenton reaction (Grose, Joss *et al.* 2006) which requires a divalent metal ion, e.g. iron, and a source of reducing equivalents to regenerate the metal, one possibility being NADH (Cabiscol, Piulats *et al.* 2000). The Fenton reaction proceeds as shown in the following equation (Imlay 2008):



The hydroxyl radicals produced via the Fenton reaction are powerful enough to react instantly with DNA, resulting in strand-breakage and/or base modification (Woodmansee and Imlay 2002).

1.12 Aims of this study

Previous experiments in our lab identified NAD-mediated SIM initially in studies of non-symbiotic *Mesorhizobium* strains (Sullivan, Eardly *et al.* 1996). These non-symbionts should not be capable of succinate utilisation as they contain non-functional *dct* genes, however, when plated under these conditions it was found that gain-of-function mutations arose in the *dct* genes which allowed the Dct symporter to function, and the organism could then utilise succinate. The occurrence of these mutations was found to be due to *nadQABC* genes present on a plasmid in these strains. Although NAD regulation in *Mesorhizobium* is an unknown mechanism, it is plausible that NADH/NADPH homeostasis is lost under stressful conditions i.e. nutrient limitation. NADH is toxic, so a build-up of this could lead to oxidative stress and a hypermutator state until a mutation allowing growth occurs, at which point the mutation will become fixed and NAD homeostasis will be restored.

The purpose of this study was to investigate the hypothesis that the apparent stress-induced Dct⁺ mutants arise as a result of oxidative DNA damage. It was hypothesised that the frequency of Dct⁺ mutants would be greatly increased in mutants deficient in the removal of ROS or repair of oxidative DNA damage in a manner dependent on the presence of the *nad* genes or high levels of NA. To investigate whether a genome-wide increase in mutation rate is occurring under these conditions, a series of single-gene knock-out mutants were constructed in components of the oxidative stress response or DNA repair systems, as well as a monofunctional catalase *katE* and a bifunctional catalase/oxidase *katG*. The mutants were tested for the generation of Dct⁺ mutants on succinate media in the presence and absence of excess NA in the R7A, R7ANS and R7AΔ*dct* backgrounds to assess the rate of reversion for each strain. Further experiments were carried out observing the emergence of rifampicin resistant colonies and several strains were assayed for their sensitivity to exogenously added H₂O₂.

2

Materials and Methods

2 Materials and Methods

2.1 Bacterial strains

M. loti and *E. coli* strains that were used in this study are listed in 2.1.

2.2 Plasmids used for this study

Plasmids used in this study are listed in

.

2.3 Media and growth conditions

E. coli was grown in Luria-Bertani (LB) or Tryptone yeast (TY) liquid medium (Miller 1972; Beringer 1974) and on LB solid agar and was incubated at either 37°C or 28°C. Mesorhizobial strains were grown in TY or *Rhizobium* defined media (RDM) (Ronson, Nixon *et al.* 1987) broths and on TY or RDM agar plates and incubated at 28°C. RDM was supplemented with the following carbon sources: 4% glucose (GRDM) or 20 mM succinate. Five% Sucrose RDM was used for selection of the markerless deletion mutant constructed using pJQ200SK. RDM contains biotin (1 µg/ml), thiamine (10 µg/ml) and NA (1 µg/ml). When excess NA is required it is added to the media at a concentration of 50 µg/ml. For further information refer to appendix 6.1.

2.4 Antibiotics

Where required, the media were supplemented with the antibiotics and fungicides listed in

Table 2.3.

2.5 Enzymes and chemicals

Enzymes were purchased from Roche Diagnostics (Mannheim, Germany) and New England Biolabs (Beverly, MA, USA). Antibiotics and chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and Roche Diagnostics (Indianapolis, USA). Antibiotics were dissolved in filter-sterile Milli-Q water and filter sterilised through a 0.45-µm syringe filter at appropriate concentrations, except for Rif and Tc, which were dissolved in 100% methanol.

Table 2.1 Bacterial strains used in this study.

Strain	Species	Characteristics	Source or reference
S17-1	<i>Escherichia coli</i>	<i>recA, thi, pro, hsdR, hasM</i> , [RP4-2(Tc::Mu)(Km::Tn7)]	(Simon, Priefer <i>et al.</i> 1983)
EPI300™	<i>Escherichia coli</i>	F ⁻ <i>mcrA</i> D(<i>mrr-hsdRMS-mcrBC</i>) f80d <i>lacZDM15</i> D <i>lacX74 recA1 endA1 araD139</i> D(<i>ara, leu</i>)7697 <i>galU galK l⁻ rpsL nupG trfA tonA dhfr.</i>	Epicentre® Biotechnologies
R7A	<i>Mesorhizobium loti</i>	Field reisolate of ICMP3153	(Sullivan, Patrick <i>et al.</i> 1995)
R7ANS	<i>Mesorhizobium loti</i>	Nonsymbiotic derivative of R7A; lacks ICEM/Sym ^{R7A}	(Ramsay, Sullivan <i>et al.</i> 2006)
R7AΔ <i>dct</i>	<i>Mesorhizobium loti</i>	R7A symbiosis island Δ <i>dctABDΩkan</i> marker exchange mutant	(Weaver 2003)
R7A- <i>katE</i>	<i>Mesorhizobium loti</i>	R7A <i>katE</i> ::pFUS2, Gm ^R	This study
R7ANS- <i>katE</i>	<i>Mesorhizobium loti</i>	R7ANS <i>katE</i> ::pFUS2, Gm ^R	This study
R7AΔ <i>dct</i> - <i>katE</i>	<i>Mesorhizobium loti</i>	R7AΔ <i>dctkatE</i> ::pFUS2, Gm ^R	This study
R7A- <i>katG</i>	<i>Mesorhizobium loti</i>	R7A <i>katG</i> ::pFUS2, Gm ^R	This study
R7ANS- <i>katG</i>	<i>Mesorhizobium loti</i>	R7ANS <i>katG</i> ::pFUS2, Gm ^R	This study
R7AΔ <i>dct</i> - <i>katG</i>	<i>Mesorhizobium loti</i>	R7AΔ <i>dctkatG</i> ::pFUS2, Gm ^R	This study
R7A- <i>mutM</i>	<i>Mesorhizobium loti</i>	R7A <i>mutM</i> ::pFUS2, Gm ^R	This study
R7ANS- <i>mutM</i>	<i>Mesorhizobium loti</i>	R7ANS <i>mutM</i> ::pFUS2, Gm ^R	This study
R7AΔ <i>dct</i> - <i>mutM</i>	<i>Mesorhizobium loti</i>	R7AΔ <i>dctmutM</i> ::pFUS2, Gm ^R	This study
R7A- <i>mutS</i>	<i>Mesorhizobium loti</i>	R7A <i>mutS</i> ::pFUS2, Gm ^R	This study

R7ANS- <i>mutS</i>	<i>Mesorhizobium loti</i>	R7ANS <i>mutS</i> ::pFUS2, Gm ^R	This study
R7AΔ <i>dct-mutS</i>	<i>Mesorhizobium loti</i>	R7AΔ <i>dctmutS</i> ::pFUS2, Gm ^R	This study
R7AΔ <i>mutT</i>	<i>Mesorhizobium loti</i>	R7A <i>mutT</i> , in-frame markerless deletion	This study
R7ANSΔ <i>mutT</i>	<i>Mesorhizobium loti</i>	R7ANS <i>mutT</i> , in-frame markerless deletion	This study
R7AΔ <i>dctΔmutT</i>	<i>Mesorhizobium loti</i>	R7AΔ <i>dctmutT</i> , in-frame markerless deletion	This study
R7A- <i>mutY</i>	<i>Mesorhizobium loti</i>	R7A <i>mutY</i> ::pFUS2, Gm ^R	This study
R7ANS- <i>mutY</i>	<i>Mesorhizobium loti</i>	R7ANS <i>mutY</i> ::pFUS2, Gm ^R	This study
R7AΔ <i>dct-mutY</i>	<i>Mesorhizobium loti</i>	R7AΔ <i>dctmutY</i> ::pFUS2, Gm ^R	This study
R7A- <i>katG</i> comp	<i>Mesorhizobium loti</i>	Strain R7A <i>katG</i> complemented with pSCRAWN1, Gm ^R /Tc ^R	This study
R7ANS- <i>katG</i> comp	<i>Mesorhizobium loti</i>	Strain R7ANS <i>katG</i> complemented with pSCRAWN1, Gm ^R /Tc ^R	This study
R7AΔ <i>dct-katG</i> comp	<i>Mesorhizobium loti</i>	Strain R7AΔ <i>dct katG</i> complemented with pSCRAWN1, Gm ^R /Tc ^R	This study

Table 2.2 Plasmids used in this study.

Plasmid	Characteristics	Source
pJQ200SK	pACYC184-derivative, suicide vector, <i>sacB</i> , Gm ^R	(Quandt and Hynes 1993)
pFUS2	Suicide vector, Gm ^R	(Antoine, Alonso <i>et al.</i> 2000)
pFAJ1708	Stable RK2-derived cloning vector containing the promoter of the <i>nptII</i> gene in the multiple cloning site, Ap ^R /Tc ^R	(Dombrecht, Vanderleyden <i>et al.</i> 2001)
pSF <i>mutT</i>	pJQ200SK containing 2400 bp fragment used to create	This Study

<i>mutT</i> mutant, cloned using XhoI/HindIII/SpeI, <i>sacB</i> , Gm ^R		
pSFkatG	pFUS2 containing an internal <i>katG</i> fragment, cloned using BamHI/HindIII, Gm ^R	This Study
pSFmutM	pFUS2 containing an internal <i>mutM</i> fragment, cloned using BamHI/HindIII, Gm ^R	This Study
pSFmutS	pFUS2 containing an internal <i>mutS</i> fragment, cloned using BamHI/HindIII, Gm ^R	This Study
pSFmutY	pFUS2 containing an internal <i>mutY</i> fragment, cloned using BamHI/HindIII, Gm ^R	This Study
pSCRAWN1	pFAJ1708 containing a copy of <i>katG</i> , Gm ^R /Tc ^R	This Study

Table 2.3 Antibiotics and fungicides used in this study.

Antibiotic	Abbreviation	Concentration (µg/ml)	
		<i>E. coli</i>	<i>Mesorhizobium</i> sp.
Cycloheximide	-	-	10
Gentamicin	Gm	25	50
Quintozone	-	-	50
Rifampicin	Rif	-	200
Tetracycline	Tc	15	2

2.6 Primers

PCR primers were designed using the DNASTar software suite. For *M. loti* strains, the primers were designed based on the genome sequence of *M. loti* MAFF303099 obtained from the website <http://genome.kazusa.or.jp/rhizobase/>. The primers designed and used in this study are listed in Table 2.4. The restriction sites are highlighted in red.

Table 2.4 PCR primers used in this study.

Primer name	Sequence	Application
KatEpFUSL	AAATTTAAGCTTCTGCTCAACGCGAAGGATGA	Left primer used for amplifying <i>katE</i>
KatEpFUSR	TTTAAAGGATCCACGTTCTGCGTCATGAACG	Right primer used for amplifying <i>katE</i>
KatGpFUSL	TTTAAAAAGCTTTACGTTCCGGCAAGACCCATG	Left primer used for amplifying <i>katG</i>
KatGpFUSR	AAATTTGGATCCTCTCATGGAAGCGCTTCGAG	Right primer used for amplifying <i>katG</i>
MutLpFUSL	TTTAAAAAGCTTGCTGCAGCAATACGCCTATG	Left primer used for amplifying <i>mutL</i>
MutLpFUSR	AAATTTGGATCCGGCCGACATAGGCGTATTGC	Right primer used for amplifying <i>mutL</i>
MutMpFUSL	TTTAAAAAGCTTTCCACCACGAGCGCTCGAA	Left primer used for amplifying <i>mutM</i>
MutMpFUSR	TTTAAAGGATCCCTTCTTGCTCGGCCTGGCGA	Right primer used for amplifying <i>mutM</i>
MutSpFUSL	TTTAAAAAGCTTGTTGACCCCTGTTTCATCGAT	Left primer used for amplifying <i>mutS</i>
MutSpFUSR	AAATTTGGATCCGCGGCGAAGATTTCGACGAT	Right primer used for amplifying <i>mutS</i>
MutSpFUSLv2	AAATTTAAGCTTGACGCGCGGCAATCTCGAAC	Left primer used for amplifying <i>mutS</i>
MutSpFUSRv2	TTTAAAGGATCCCATCGCCTCCGCCAACTCCT	Right primer used for amplifying <i>mutS</i>
MutTpJQLL	TTAACTAGTCGCAAGCAGGTCGAAGTCAC	Left primer used for

		amplifying <i>mutT</i> left arm
MutTpJQLR	ATAAATT AAGCTT GCCGGCATTGGCCAGAT	Right primer used for amplifying <i>mutT</i> left arm
MutTpJQRL	AATATAT AAGCTT ACCTTCGCCAGCCATTC	Left primer used for amplifying <i>mutT</i> right arm
MutTpJQRR	AATT CTCGAG AGACCGTGACGGTGCGCTAT	Right primer used for amplifying <i>mutT</i> right arm
MutTseqcheckrev1	AGCGACGATCAGGCCATATT	<i>mutT</i> Insert Check
MutTseqcheckrev2	TGCCATCGGAGCTGATGATG	<i>mutT</i> Insert Check
MutTseqcheckfwd1	CTGGAGTCGGTTCGACGAAT	<i>mutT</i> Insert Check
MutTpJQcheckshortL	AGCAGCGTCACGCGCAGAAT	<i>mutT</i> Insert Check
MutTpJQcheckshortR	ACGCTGATCTGCGTG CATGA	<i>mutT</i> Insert Check
MutYpFUSL	AAATTT AAGCTT TCTGGCTGTCCGAAGTCATG	Left primer used for amplifying <i>mutY</i>
MutYpFUSR	AAATTT GGATCCT TCGTTGAGTGGCGTCCTGA	Right primer used for amplifying <i>mutY</i>
KatGcompL	TTTAAA AAGCTT TCAGTCATGCGCACCGACTT	Left primer used for complementation of <i>katG</i>
KatGcompR	AAATTT AAGCTT TCTGGATCAGCCGGCGAT	Right primer used for complementation of <i>katG</i>
KatGseqL1	GCACGCGCTGATGACGGATT	<i>katG</i> sequence left primer
KatGseq2	GGCCAGAACGGAACGTCGAC	<i>katG</i> sequence right primer

2.7 Polymerase Chain Reaction (PCR)

The PCR reactions were carried out using the Phusion™ High-Fidelity PCR kit (Finnzymes, cat # F-553S). The reaction mixtures were made to a volume of 100 µl and consisted of the following: 20 µl of 5x high fidelity buffer, 2 µl of dNTP mix (10 mM), 3 µl of dimethyl sulfoxide (DMSO), 1 µl (approximately 20 ng) of template DNA, 1 µl of each primer, 0.5 µl of Phusion™ enzyme (2,000 units/ml) and 72.5 µl of filter-sterile MilliQ H₂O. The reactions were performed in a Hybaid PCR Express Thermal Cycler using a standard protocol. This protocol consisted of the first cycle at 98°C for 1 min; the second cycle was 98°C for 10 sec, 57°C for 15 sec and then 72°C for 20 sec, which was repeated 20 times; the final cycle was 98°C for 10 sec, 57°C for 15 sec and 72°C for 5 min. In some instances where PCR products were difficult to obtain, PCR reactions were run in triplicate with a gradient of annealing temperatures ranging from 54°C to 62°C for the second part of cycle two. The PCR products obtained were purified with the High Pure PCR Product Purification Kit (Roche, cat # 1732688), performed as detailed in the instructions provided.

2.8 DNA isolation and preparation

2.8.1 Genomic DNA extractions

2.8.1.1 Ultra-quick genomic DNA preparations

DNA isolation for *Mesorhizobium* sp. was carried out using a modified protocol of the ultra-quick genomic DNA preparation described by (Gonzalez-y-Merchand, Colston *et al.* 1996). Five ml TY cultures were grown for 48-72 hours at 28°C with shaking. Three ml of culture was harvested by centrifugation at 14,926 g and supernatant was discarded. Cells were re-suspended in 400 µl lysis buffer (4 M guanidinium thiocyanate, 10 mM EDTA, 0.1% [w/v] tween-80). The cell suspension was snap-frozen (-70°C) in a dry ice/ethanol bath for 15 min. The suspension was then incubated at 65°C for 5 min. This snap-freezing/heating process was repeated twice. The bacterial lysate was then extracted, first using phenol-chloroform followed by a 5 min spin at 14,926 g. Then extracted again using chloroform followed by a 3 min spin at 14,926 g. The DNA was then precipitated using 1 ml of ethanol plus 50 µl of 3 M sodium acetate, tubes were inverted and then centrifuged at 14,926 g for 10 min. The final wash was carried out with 1 ml of 70% ethanol and the pellet was dried and re-suspended in 100 µl of filter-sterile Milli-Q water and incubated at 37°C for 30 min.

2.8.1.2 PrepMan™ Ultra genomic DNA preparations

Genomic DNA for use in PCR was extracted from bacterial pellets, harvested from 1 ml of culture. The cells were re-suspended in 200 µl of PrepMan™ Ultra reagent (Applied Biosystems) and boiled for 10 min before being ice-cooled for two min and then centrifuged at 14,926 g for three min. One µl of the resulting supernatant was used in PCR reactions.

2.8.2 Plasmid DNA extractions

2.8.2.1 Alkaline lysis preparations

Alkaline lysis minipreps were carried out via the protocol described by Feliciello and Chinali (Feliciello and Chinali 1993). Overnight cultures were grown in LB broth at 37°C and were harvested by centrifugation at 14,926 g. This was carried out twice so that 3 ml of culture was pelleted into a microfuge tube. Cell pellets were re-suspended into 250 µl of lysis solution (0.2 M sodium hydroxide [NaOH], 1% [w/v] sodium dodecylsulphate [SDS]) with a 2 min incubation. A volume of 350 µl of neutralising solution (2 M potassium acetate/1 M acetic acid) was added and mixed by inversion, followed by a 10 min centrifugation at 14,926 g. From this tube, 800 µl of supernatant was transferred to a new tube where the DNA was precipitated with the addition of 560 µl of isopropanol. The tube was then centrifuged at 14,926 g for 10 min and the supernatant discarded. The DNA pellet was washed by adding 1 ml of 70% ethanol, before a 5 min spin. The last supernatant was removed and the tube was air-dried before the pellet was re-suspended in 50 µl of filter-sterile Milli-Q water.

2.8.2.2 Plasmid isolation using a commercial kit

High-quality DNA preparations for cloning and sequencing was isolated using the High Pure Plasmid Isolation Kit (Roche, cat # 11754777001).

2.9 Restriction digests

Plasmid DNA preparations were digested using one or more of the restriction endonucleases: BamHI, HindIII, XhoI, PstI and SpeI in the appropriate buffers according to the manufacturers recommendations (Roche and New England Biolabs). The reaction mixture for PCR products consisted of 44 µl of product, 5 µl of the appropriate buffer and 1 µl for each restriction enzyme. For DNA preparations the reaction mixture consisted of 15 µl of DNA, 6 µl of the appropriate buffer, 1 µl of each restriction enzyme required and the rest of the volume made up to 60 µl using filter-sterile MilliQ H₂O. All digests were incubated at 37°C for at least 1.5 h.

2.10 Agarose gel electrophoresis

DNA digests were mixed with bromophenol blue tracking dye and separated on 1% agarose gels. The agarose gels were made using 1x Tris-acetate EDTA (TAE) buffer containing 1 µg/ml of ethidium bromide which was also used as the buffer they were run in (Appendix 6.2). The marker used is a mix of lamda DNA HindIII digest and bacteriophage ϕ X174 DNA HaeIII digest. Gels were run for approximately 1 hour on 90 – 100 V, except for gels used for southern blotting which were run at 25 V overnight. Gel photos were captured with a Gel Logic 200 Imaging System using UV illumination.

2.11 Ethanol precipitation

Ethanol precipitation was carried out on DNA samples by adding 2.5 x volumes of 100% ethanol plus 0.1 x volumes of sodium acetate (3 M NaAc) and 1 µl of pellet paint. These reagents were mixed then centrifuged for 10 mins at 14,926 g. The supernatant was discarded and then 70% ethanol was added before centrifuging for 5 mins at 14,926 g. Ethanol was removed (using pipette if required) before air-drying in a 37°C incubator for 10 mins. The pellet was then resuspended in 5 µl of filter-sterile MilliQ H₂O.

2.12 Subcloning of DNA fragments

2.12.1 Vector and insert DNA preparation

Vector and insert DNA was digested using the appropriate restriction enzymes. The vector or insert DNA was then prepared for ligation by using either the High Pure PCR product purification kit (Cat. # 1 732 688, Roche Diagnostics) or the QUIAX-II gel extraction kit (QUIAGEN) according to the manufacturers instructions. Dephosphorylation of the vector DNA was carried out by the addition of 2 µl alkaline phosphatase (1 U/µl) (Cat. # 713 023, Roche Diagnostics) followed by a 37°C incubation for 30 min.

2.12.2 Ligations

Ligation mixtures consisted of 3 µl of vector DNA, 14 µl of insert DNA, 2 µl of 10 x ligation buffer and 1 µl of T4 DNA ligase (Roche) to make a volume of 20 µl, which were incubated at 12°C overnight.

2.12.3 Electrocompetent cell preparations

2.12.3.1 *Escherichia coli*

Electrocompetent cell stocks of *E. coli* S17-1λ and EPI300™ were prepared using a method adapted from Sheng *et al* (Sheng, Mancino *et al.* 1995). Strains were grown in 5 ml LB broth

until they reached stationary phase, then 0.5 ml was used to inoculate 500 ml LB broths that were incubated at 37°C with shaking at 200 rpm. Cell cultures were checked using a spectrophotometer at OD₆₀₀ for a density of 0.6 – 0.8. Cells were then harvested at 4°C by centrifugation at 5930 g for 10 min. After two washes in chilled 10% [w/v] glycerol, cells were washed using 30 ml chilled 10% [w/v] glycerol, pelleted, and re-suspended in 1 ml of 10% [w/v] glycerol. The cells were then aliquotted into 40 µl volumes and snap-frozen in a dry ice/ethanol bath and stored at -80°C.

2.12.3.2 *Mesorhizobium* spp.

Electrocompetent *Mesorhizobium* spp. cells were prepared using a method developed by Kazuhiko Saeki (John T. Sullivan, personal communication). Cultures of strains were incubated to stationary phase in 5 ml TY broth. One ml of this culture was used as a seeder broth to inoculate 200 ml TY broths which were incubated at 28°C with shaking. The cells were harvested at OD₆₀₀ 0.1 – 0.3 by centrifugation at 4°C for 10 min at 5930 g. The pelleted cells were then washed using chilled 10% glycerol in a volume of 200 ml and centrifuged. This was repeated using a volume of 100 ml and then again with a volume of 4 ml of 10% glycerol. The cells were then resuspended in 200 µl of 10% glycerol, aliquotted at 40 µl volumes and snap-frozen in a dry ice/ethanol bath before being stored at -80°C.

2.12.4 Electrotransformation

Electrocompetent *E. coli* cells were prepared using the protocol described by Sheng *et al.* (Sheng, Mancino *et al.* 1995). The 40 µl aliquots of competent cells were thawed and 2 µl of DNA was added. This was then transferred to a Bio-Rad Gene Pulser® cuvette that had been chilled on ice which was subsequently transformed at 1800 V using a Bio-Rad Gene Pulser Xcell™ Electroporation System. One ml of LB broth was added to the cuvette immediately after the electroporation, which was subsequently transferred to a sterile bijoux and incubated at 37°C with shaking for 45 mins.

2.13 Bacterial crosses

2.13.1 Spot matings

2.13.2 Bi-parental matings

Bi-parental spot matings were carried out using the protocol described by Ramsay (Ramsay 2008). In each mating, three *M. loti* recipient strains were used: R7A, R7ANS and R7AΔ*dct*. These were grown in 5 ml TY broths at 28°C for 48 hours with shaking. The donor cells, *E. coli* S17-1/pJQ200SK and S17-1/pFUS2 were grown in 5 ml TY broths at 37°C for 24 hours with

shaking. Twenty five μl aliquots of a particular donor and recipient culture were overlaid to form a 50 μl aliquot on TY agar. These were dried using a laminar flow cabinet before being incubated for 24 hours at 28°C. Using a sterile loop, the spot growth was scraped off and transferred to GRDM agar plates containing the appropriate antibiotics. Single colonies were passage-purified three more times to ensure uncontaminated pure colonies were obtained.

2.13.3 Tri-parental matings

Tri-parental spot matings were carried out as is detailed in section 2.13.2. The difference was that in this instance, a helper strain was added as a third spot. Thirty μl of donor cells, 30 μl of recipient cells and 30 μl of a helper strain were overlaid to form a 90 μl aliquot on TY.

2.14 Spectroscopy

The optical density of cultures was determined using a Jenway 6300 spectrophotometer (Jenway Instruments) at OD₆₀₀ nm wavelength. DNA concentrations were measured using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies).

2.15 DNA sequencing

DNA sequencing was performed at the Allan Wilson Centre Genome Service at Massey University (Palmerston North, NZ). The reaction mixtures prepared contained the following: 300 ng of DNA (Based on nanodrop reading), 1 μl of primer (diluted to 3 pmol/15 μl). The total volume was 15 μl and the remaining volume was made up with filter-sterile MilliQ H₂O.

2.16 Computer analysis of DNA sequence data

Bioinformatic analysis was carried out using the software Seqman and Editseq, which are part of the DNASTAR lasergene® core suite. Sequence chromatograms were viewed using 4Peaks. The National Centre for Biotechnology Information (NCBI) database was used as a search tool for similar amino acid and nucleotide sequences using Blast (<http://blast.ncbi.nlm.nih.gov/>). Rhizobase (the genome database for rhizobia) was used for searching and identifying amino acid and sequence information for *Mesorhizobia* spp (<http://genome.kazusa.or.jp/rhizobase/>).

2.17 Mutagenesis

2.17.1 Insertional Duplication mutagenesis (IDM)

IDM mutants were created in *M. loti* R7A, R7ANS and R7A Δ dct using the suicide vector pFUS2 (Antoine, Alonso *et al.* 2000). Intragenic regions of target gene sequence (~350 bp) were PCR amplified with HindIII and BamHI restriction sites incorporated. The resulting PCR products

were ligated into pFUS2 which was transformed by electroporation into *E. coli* S17-1λ cells. Spot-matings were carried out between each of the three different *M. loti* strains and the *E. coli* cells containing the mutant construct. After the spot matings were carried out the cells were single colony purified three times on G/RDM containing Gm.

2.17.2 Markerless deletion mutagenesis

Primers were designed to amplify 1.2-Kb PCR fragments left and right of the target gene. The outermost end of the left arm was designed to incorporate a *Spe*I site and the outermost end of the right arm was designed with an *Xho*I site; the inner end of both products incorporated a *Hind*III site. A three-way ligation was carried out that fused the two products via the *Hind*III sites into suicide vector pJQ200SK, (Quandt and Hynes 1993) which had been digested with *Spe*I and *Xho*I. The recombinant plasmid was electroporated into *E. coli* S17 cells then mated into *M. loti* R7A, R7ANS and R7AΔ*dct* via bi-parental spot-matings as detailed in section 2.13.2. The vector contains Gm^R and colonies exhibiting this phenotype were single colony purified and confirmed using PCR. To select for the second crossover event, which results in the loss of pJQ200SK and the wild-type copy, the Gm^R colonies were passaged onto sucrose RDM (5% sucrose). The vector contains *sacB* which is lethal in the presence of sucrose so only colonies that have dropped out pJQ200SK can grow. PCR was used to confirm the success of the second crossover and the resulting markerless deletion strain.

2.18 Southern Hybridisation

Recipes for the buffers and solutions utilised in this section are provided in appendix 6.2.

2.18.1 Downward capillary transfer

Genomic DNA is run overnight in a 1% agarose gel and is transferred to Hybond™-N⁺ nylon transfer membranes (GE Healthcare) by capillary action.

2.18.1.1 Gel pre-treatment

The agarose gel containing the genomic DNA to be transferred is run through a series of washes to prepare it for transfer. The gel is soaked in depurination solution (0.25 M hydrochloric acid [HCl]) for 15 min and rinsed twice in distilled water (dH₂O). The gel is then immersed in denaturation solution (1.5 M sodium chloride [NaCl], 0.4 M NaOH) for 20 min, and this step is repeated. This is followed by a final soak in neutralisation solution (1.5 M NaOH, 0.5 M Tris [pH 8.0]) for 20 min.

2.18.1.2 Blot Assembly and transfer

For the downward capillary transfer to be carried out, a stack had to be prepared. It consisted of the following, from the base upwards: A 6 cm stack of paper towels, 4 dry pieces of Whatman filter paper, 1 wet (soaked in 20 x SSC) piece of Whatman filter paper. The membrane (rinsed in dH₂O, then rinsed in 20 x SSC for 5 min) is situated on the Whatman paper and the gel (with the wells removed) was placed on top of the membrane, ensuring no air bubbles were present. The final components of the stack consist of three more wet pieces of Whatman paper, two of which have an extension forming a bridge into a container of 20 x SSC. This stack was left assembled for a minimum of three hours to allow sufficient transfer of the DNA to the membrane.

2.18.1.3 Alkali fixation

The Southern blot stack was disassembled after sufficient time for transfer to have occurred. The membrane was placed DNA-side up on a wet (soaked in 0.4 M NaOH) piece of Whatman filter paper, before extra 0.4 M NaOH was poured directly over the membrane and left for 20 min. The membrane was then rinsed in 20 x SSC, before being placed on a dry piece of Whatman filter paper and allowed to air-dry. The confirmation of DNA transfer was assessed visually using a UV-transilluminator by examining fluorescence.

2.18.2 Hybridisation of probe to membrane-bound DNA

2.18.2.1 Prehybridisation

Prehybridisation of the membrane was carried out using 16.5 ml (0.125 ml/cm² of membrane) of AlkPhos Direct™ hybridisation buffer (GE Healthcare) rotating at 55°C in a Hybaid bottle for 15 min.

2.18.2.2 Probe preparation

The appropriate probe DNA (PCR product or vector DNA) was diluted to a concentration of 10 ng/μl in water, which was supplied in the AlkPhos Direct™ labelling system. Ten μl of the DNA dilution, plus 1 μl of λ DNA (Mix of lamda DNA HindIII digest and bacteriophage φ X174 DNA HaeIII digest) was added to a 0.5 ml microfuge tube and boiled for five min. This tube was then placed on ice to cool for five min, spun briefly and placed back on ice. The following reagents were added to the tube: 10 μl of reaction buffer, 2 μl labelling reagent and finally 10 μl of working-concentration (20 μl of crosslinker mixed with 80 μl water) crosslinker solution. The tube was mixed thoroughly but gently between each addition. Once all reagents were added the tube was spun briefly, before being placed at 37°C for 30 min.

2.18.2.3 Hybridisation

After the prehybridisation period, 5 – 10 ng of probe per ml of buffer is added to the AlkPhos Direct™ hybridisation buffer. It was important not to add the probe directly to the membrane so 5 ml of buffer was removed and the probe was mixed into this solution. The 5 ml of buffer containing the probe was returned to the Hybaid bottle and this was then placed into the hybridisation oven. This was left for 12 – 18 hours rotating at 55°C.

2.18.2.4 Membrane washing

Following hybridisation, membranes were washed with heated (55°C) primary wash buffer using two – five ml per cm² of membrane for 10 min with gentle agitation, twice. The membrane was transferred to a clean container and an excess of secondary wash buffer was added, and left for 5 min with gentle agitation. This was repeated with fresh secondary wash buffer.

2.18.2.5 Signal generation/detection

The secondary wash buffer is drained from the membrane using tweezers, the membrane was then placed DNA-side up onto gladwrap without letting the membrane dry out. Two ml of detection reagent was squirted across the membrane and left for two – five min. Excess reagent was then drained and the membrane was wrapped in the gladwrap and exposed to X-Ray film (Cronex, Dupont) for 2-72 h in the presence of Quanta-rapid or Lightening-plus intensifying screens.

2.18.3 Membrane stripping

When a labelled membrane was required for a subsequent hybridisation the probes were removed from the membrane using 0.5% [w/v] SDS solution. The SDS solution was boiled and then added to the membrane for 30 min with shaking. Once complete, the membrane was rinsed in 2 x SSC, air-dried and stored between two sheets of Whatman filter paper at room temperature until needed.

2.19 Viability curve

Three five ml TY broths were inoculated for each of the strains of interest and incubated at 28°C with shaking at 160 rpm for two days. Two ml of the seeder broth was added to 250 ml flasks containing 100 ml of TY broth. The cultures were grown at 28°C with shaking at 160 rpm for 150 h. The cultures were sampled at time zero, and then three times daily. At each sample point the OD_{600 nm} was recorded and cfu/ml was calculated by plating out duplicate dilution samples onto G/RDM. Each sample was run as biological triplicates.

2.20 Hydrogen peroxide sensitivity assay

Approximately 1.0×10^8 *M. loti* cells (grown overnight) were mixed with 10 ml of molten top agar (TY containing 0.7% agar) which was pre-warmed to 42°C. This was overlaid onto pre-warmed (28°C) TY agar plates (85 mm diameter) containing Gm. The top agar was allowed to solidify before adding six mm discs, prepared from Whatman filter paper, which contained seven μ l of 0.61 M H_2O_2 to the centre of the plate. Zones of growth inhibition were measured in mm after 4 days of incubation at 28°C. This protocol was modified from Hanyu *et al*, 2009.

2.21 Dct reversion assay

Fresh plates of the particular strain of interest were prepared and used to inoculate 5-ml TY broths which were incubated for 72 hours. The broth cultures were adjusted using sterile TY medium to an OD_{600} of 1.0 ± 0.1 in order to standardise the initial dilution. A 10-fold dilution series was carried out from 10^0 through to 10^{-6} in MilliQ H_2O and 0.1 ml of each dilution was plated onto succinate RDM and incubated at 28°C for ~15 days. Dct⁺ colonies were counted daily and recorded in a cumulative fashion. The dilution plated at 10^{-6} was only counted once, before any Dct⁺ colonies arose, and was used to calculate the cell titre for each sample to ensure similar cell numbers between data sets. This process was carried out in triplicate for each strain tested to ensure statistical significance.

2.22 Rifampicin mutagenesis assay

This experiment was carried out in the same manner as the Dct⁺ reversion assay detailed in Section 2.21. The only difference was that in this case, instead of being plated on succinate RDM, the strains were plated onto G/RDM containing the antibiotic Rifampicin at a concentration of 200 μ g/ml.

3

Results

3 Results

3.1 R7A Δ dct and R7ANS phenotypes

M. loti R7A contains two copies of the *dct* locus, one within the symbiosis island and the other on the chromosome. The chromosomal copy of the *dct* locus is cryptic, meaning the genes are intact but non-functional. The wild-type *M. loti* R7A contains the symbiosis island with the functional *dct* locus (Sullivan, Trzebiatowski *et al.* 2002) which means it is able to utilise C4-dicarboxylates, e.g. succinate, as a sole carbon source (Dct⁺ phenotype). Strains that do not contain a copy of this locus, e.g. *M. loti* R7A Δ dct, are unable to utilise succinate as a sole carbon source (Dct⁻ phenotype). However when strain R7A Δ dct is plated on solid medium containing succinate, Dct⁺ colonies begin to arise after approximately 5-7 days incubation depending on the number of cells plated. The Dct⁺ colonies are large (3-4 mm) compared to the background microcolonies (≤ 1 mm) and continue to appear up to ≈ 25 -30 days post inoculation on plates with a lower number of cells plated. The number of Dct⁺ colonies that appear is dependent on the dilution series plated; however at the lower dilutions, the total number of Dct⁺ colonies is similar due to the lack of nutrients available at such high cell numbers (Ashbrook 2012). *M. loti* R7ANS is a derivative of strain R7A that lacks the symbiosis island but still contains the cryptic *dct* genes. This strain when plated on succinate does not give rise to any Dct⁺ colonies. This result suggests that the mobile symbiosis island contains a factor(s) that is responsible for the appearance of the Dct⁺ phenotype. Previous work identified this factor as the *nadABC* operon that is required for the synthesis of nicotinamide, an intermediate in the synthesis of NAD (Ashbrook 2012). Strains lacking the operon require nicotinamide or NA for growth, as the strains can convert NA to nicotinamide.

In the non-symbiotic strains exogenous NA can confer the mutator phenotype, presumably by providing an increase in NAD generated through the NAD salvage pathway. Previous work (Ashbrook 2012) showed that when a non-symbiotic strain was grown on medium containing 1 μ g/ml of NA, virtually no Dct⁺ colonies arise. However, when an excess of NA (50 μ g/ml or greater) was added, the same strain gave rise to a large number of the Dct⁺ colonies. Although there is no *nadABC* locus in the R7ANS strain present to produce NAD through the *de novo* biosynthetic pathway, the salvage pathway still exists and this would explain how an increase in intracellular NAD could be achieved by the addition of 50 μ g/ml of NA to the medium. Therefore experiments involving mutants of strain R7ANS described below were carried out in the presence of both 1 μ g/ml and 50 μ g/ml NA.

To establish a base-line for the current study, cells from a 48-h TY culture of R7A Δ dct were plated out onto succinate-containing medium with 1 μ g/ml NA to observe Dct⁺ “revertant” colonies, in a 10-fold dilution series ranging from 10⁰ through to 10⁻⁵ (Fig. 3.1). The results were similar to those of Ashbrook (2012) described previously, with the main difference between dilutions being the rate at which revertant colonies arose (Fig. 3.1). This observation is thought to reflect a simple density-dependent effect caused by the larger number of cells at the lower dilution, leading to exhaustion of trace carbon in the medium at a faster rate, and therefore pushing the cells into starvation stress and hypermutation sooner than the cells plated at a higher dilution.

When scoring for Dct⁺ revertants on succinate-containing media in later experiments, various dilutions were plated. However to reduce the number of graphs, only the data from plating the 10⁻³ dilution (about 10⁶ cells) have been shown in most cases. This was done simply to improve clarity of the graphs as each dilution gave the same overall trend, as shown in Fig. 3.1.

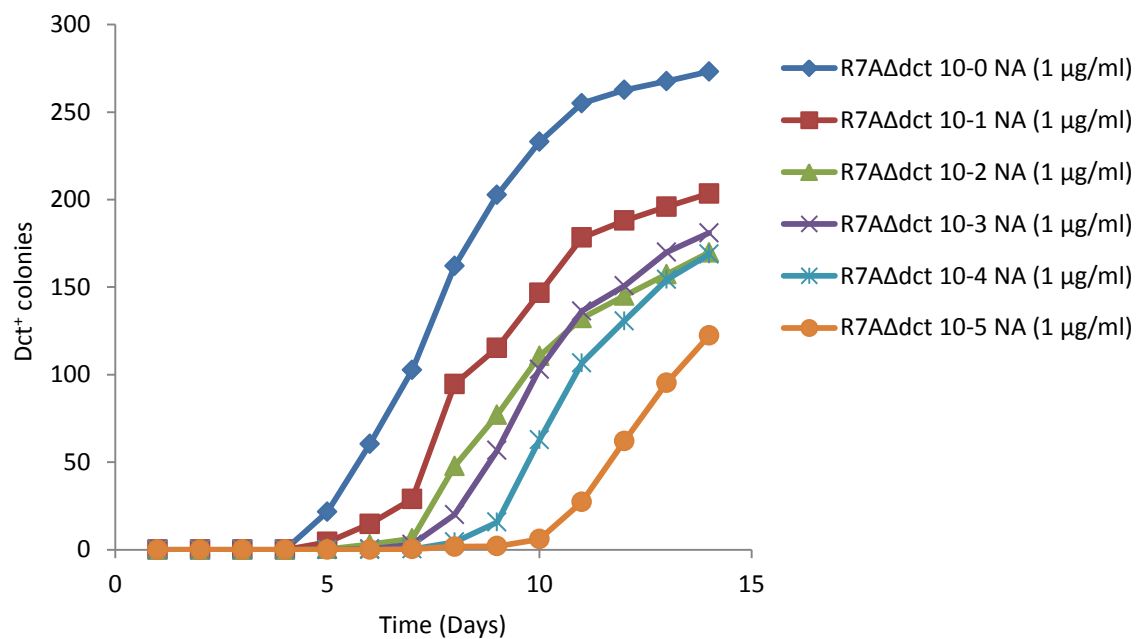


Figure 3.1 Appearance of Dct⁺ colonies over time in strain *M. loti* R7A Δ dct in a 10-fold dilution series. This shows the cell-density-dependent effect on the rate at which Dct⁺ colonies arise on succinate medium containing 1 μ g/ml NA. The TY culture that was plated contained 9.8×10^8 cfu/ml as calculated from the number of microcolonies formed on the 10⁻⁶ dilution.

3.2 Selection of target genes

The first aim of this project was to identify oxidative stress response and DNA repair genes present in *M. loti* R7A. As described below, the following genes were selected: DNA mismatch repair (*mutS* and *mutL*), formamidopyrimidine-DNA glycosylase (*mutM*), dGTP pyrophosphohydrolase (*mutT*), adenine glycosylase (*mutY*), mono-functional catalase (*katE*) and bi-functional catalase/peroxidase (*katG*). These were chosen to construct single gene knock-out mutants in three different backgrounds: *M. loti* R7A, *M. loti* R7ANS and *M. loti* R7A Δ dct. This was achieved by IDM using the suicide vector pFUS2 for all but the *mutT* mutant, which was constructed via the markerless deletion method. The pFUS2 mutants were constructed as transcriptional fusions to a promoterless *lacZ* reporter gene. The inactivation of the chosen gene present in the host strain chromosome occurred through integration via homologous recombination. Once each of the mutants was confirmed, their particular phenotypes were analysed and compared with the corresponding wild-type strain.

3.2.1 Sequence similarity between *M. loti* strain R7A and *M. loti* strain MAFF303099

At the time when primers were being designed, the genome of *M. loti* R7A had yet to be fully sequenced, with only the symbiosis island sequence available (Sullivan, Trzebiatowski *et al.* 2002). The strain *M. loti* MAFF303099 has been fully sequenced (Kaneko, Nakamura *et al.* 2000) and sequencing data of various genes from R7A in previous studies indicated approximately 98% sequence identity between the strains. Due to this sequence similarity, *M. loti* MAFF303099 sequence was used for the selection of potential genes that should be present in the R7A genome as well as the template for the design of primers (Table 2.4).

3.2.2 Selection of target genes

The genes of interest chosen for this study included a series of *mut* genes involved in DNA mismatch repair and GO repair systems. Two genes, *katE* and *katG*, which had been characterised for *M. loti* strain MAFF303099 (Hanyu, Fujimoto *et al.* 2009) and protect against reactive oxygen species, were also chosen. BlastP searches were performed on the rhizobase website (<http://genome.kazusa.or.jp/rhizobase/>) using protein sequences encoded by a selection of *E. coli* K12 genes as search queries to identify the corresponding genes in *M. loti* strain MAFF303099. The genes investigated are listed in Table 3.1. As noted above, the final genes selected for this study were the MMR genes *mutL* and *mutS*, the GO repair system genes

mutM, *mutT* and *mutY*, and the catalase/peroxidases *katE* and *katG*. The other genes considered were omitted either due to a lack of a convincing homologue in *M. loti*, or gene redundancy that could potentially mask the effects of a single gene knockout.

Table 3.1: Genes investigated as potential mutagenesis targets in this study

Gene Name*	Name in <i>M. loti</i> MAFF303099	Protein length (a.a) in <i>E. coli</i> K12 MG1655	Protein length (a.a) <i>M. loti</i> MAFF303099	% ID in <i>E. coli</i> K12 MG1655	Gene function
<i>mutS</i>	<i>mll5328</i>	854	909	40%	Methyl-directed mismatch repair
<i>mutL</i>	<i>mll8278</i>	616	627	36%	Methyl-directed mismatch repair
<i>mutM</i>	<i>mll5585</i>	270	296	39%	Formamidopyrimidine-DNA glycosylase
<i>mutT</i>	<i>mll3459</i>	130	140	30%	Nucleoside triphosphate pyrophosphohydrolase
<i>mutY</i>	<i>mll7523</i>	351	396	37%	Adenine DNA Glycosylase
<i>katG</i>	<i>mlr6940</i>	727	756	62%	Catalase/peroxidase
<i>katE</i>	<i>mlr2101</i>	754	702	49%	Catalase
<i>rpoE</i>	<i>mll8140</i>	192	208	31%	RNA polymerase, sigma 24 (sigma E) factor
<i>soxR</i>	<i>mlr2720</i>	155	148	65%	DNA-binding transcriptional dual regulator
<i>xthA</i>	<i>mll2070</i>	269	256	32%	Exonuclease III
<i>oxyR</i>	<i>mll6938</i>	306	302	36%	DNA-binding transcriptional dual regulator
<i>uvrD</i>	<i>mlr1503</i>	721	817	43%	DNA helicase II
<i>mutH</i>	<i>mlr6241</i>	230	329	26%	Methyl-directed Mismatch repair

* Genes selected are shown in red.

3.3 Construction of IDM mutants

All of the IDM mutants constructed in this study were made using the protocol outlined in Fig. 3.2.

3.3.1 Construction of *katE*, *katG*, *mutM*, *mutY*, *MutL* and *mutS* IDM pFUS2 vectors

In-frame transcriptional *lacZ* fusion mutants were constructed using the IDM process. The suicide vector pFUS2 (Antoine, Alonso *et al.* 2000) was selected as it contains gentamicin resistance (Gm^R), as well as a promoterless *lacZ* reporter gene, and is unable to replicate in *M. loti*.

The mutants were constructed by first cloning short intragenic regions (approximately 350-400 bp) of the selected target gene into pFUS2 as shown in Fig 3.2. The size of the fragments chosen was large enough to allow homologous recombination, the frequency of which is proportional to the length of the fragment, but not too large so as to reconstitute the gene after insertion. The intragenic regions were PCR amplified (Figure 3.3), and these products were digested using restriction endonucleases compatible with pFUS2, allowing ligation of the products into pFUS2. Confirmation of the constructs was carried out using restriction analysis and DNA sequencing. Plasmid DNA from *E. coli* clones representing each construct was extracted using the alkaline lysis method (section 2.8.2.1). Restriction analysis was performed on the extracted DNA using the appropriate endonucleases, to show that the desired PCR product had successfully ligated into pFUS2. Digestion of the clones was expected to reveal a pFUS2 band of 7.2 kb and a PCR product of approximately 350 bp (Figure 3.4).

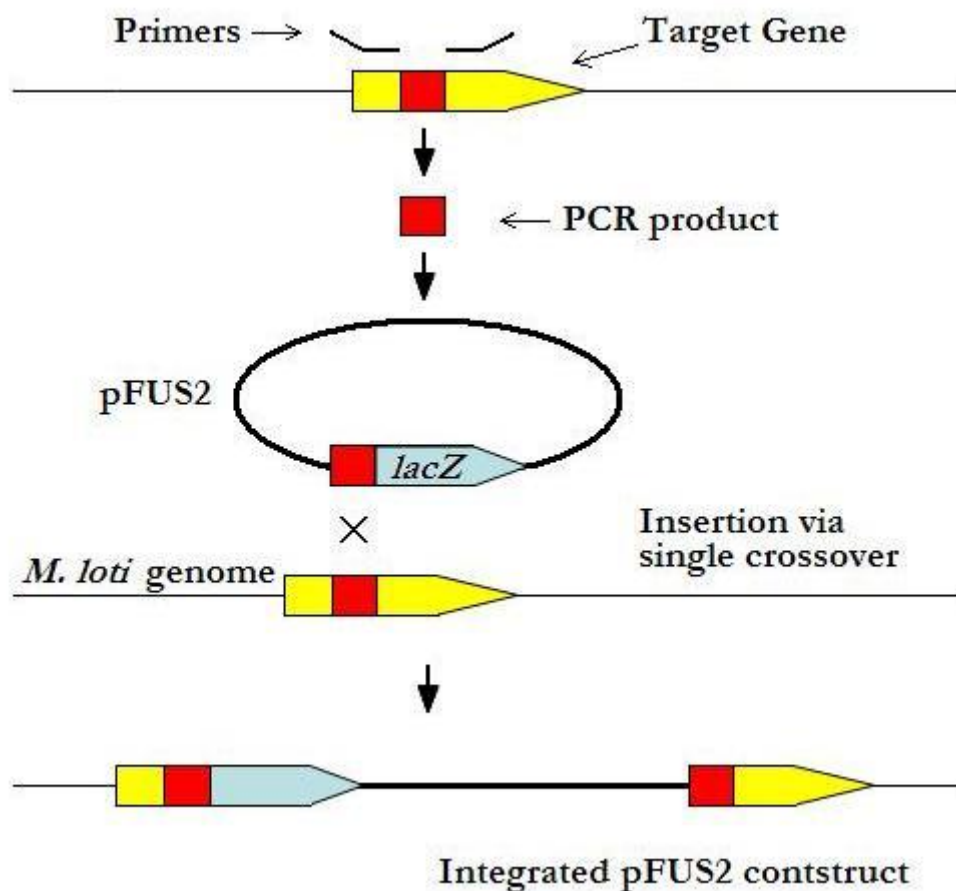


Figure 3.2 Schematic representation of the IDM method used to construct mutants using the suicide vector pFUS2. Internal regions of approximately 350 bp of *mutL*, *mutM*, *mutS*, *mutY*, *katE*, and *katG* were PCR amplified with flanking restriction endonuclease sites. The PCR products were cloned into pFUS2 and then transformed into *E. coli* S17-1 λ pir electrocompetent cells. The pFUS2 construct was then introduced to the *M. loti* chromosome via conjugation followed by homologous recombination. The result is a single gene disruption due to integration of the pFUS2 construct.

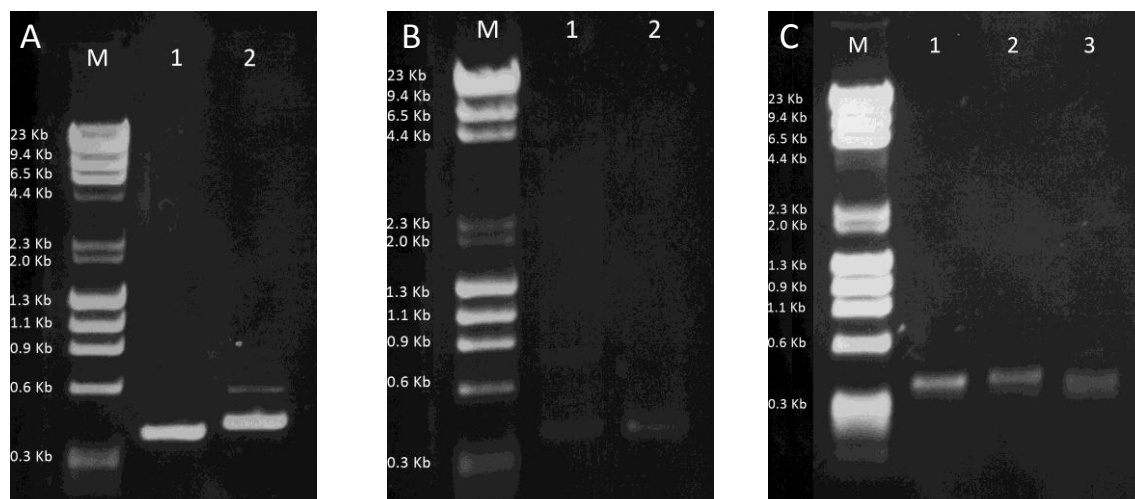
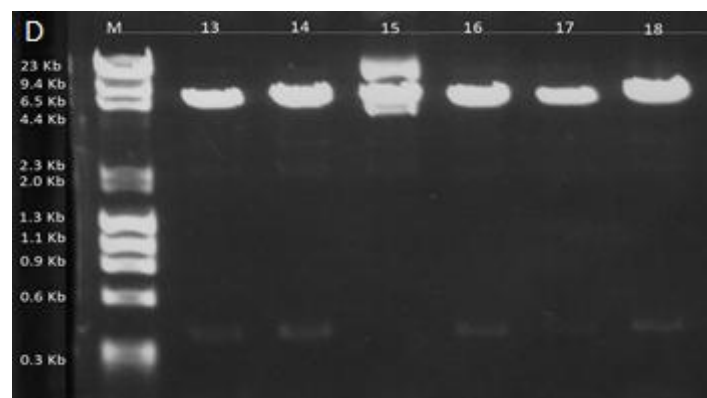
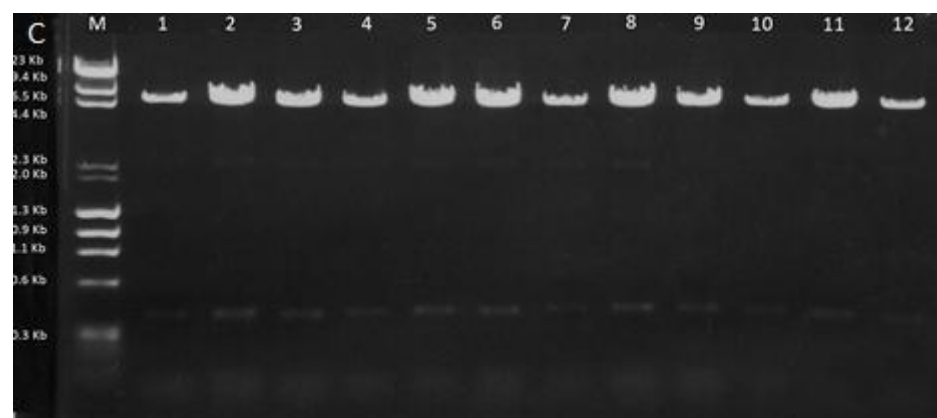
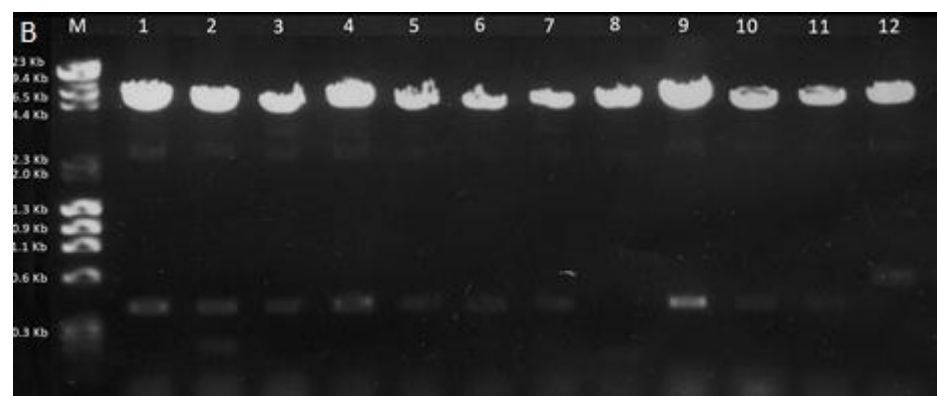
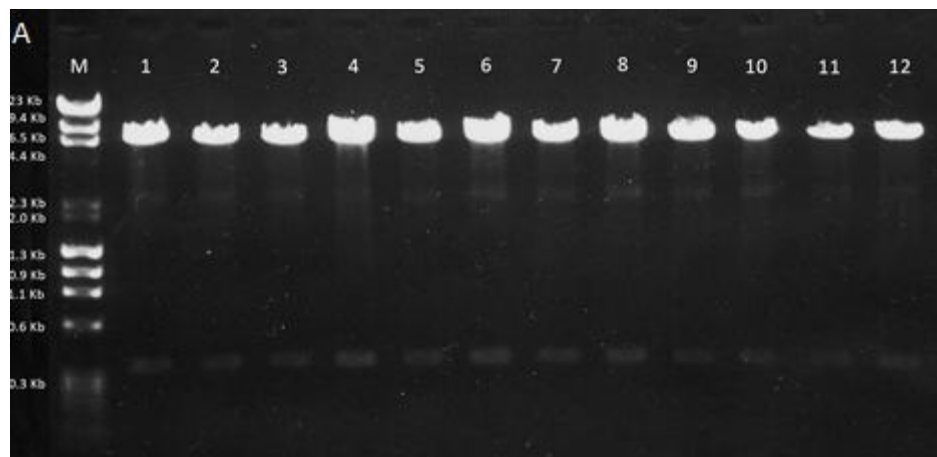


Figure 3.3 Agarose gels showing PCR products. Lane M represents λ HindIII/ ϕ X174 HaeIII DNA size marker. A) Lane 1 represents PCR product of *katE*; Lane 2 represents PCR product of *katG*. B) Lane 1 and 2 represent PCR products of *mutL* using different primer pairs. Lane 2 shows the successful product. C) Lane 1 represents PCR product of *mutM*; Lane 2 represents PCR product of *mutS*; Lane 3 represents PCR product of *mutY*.



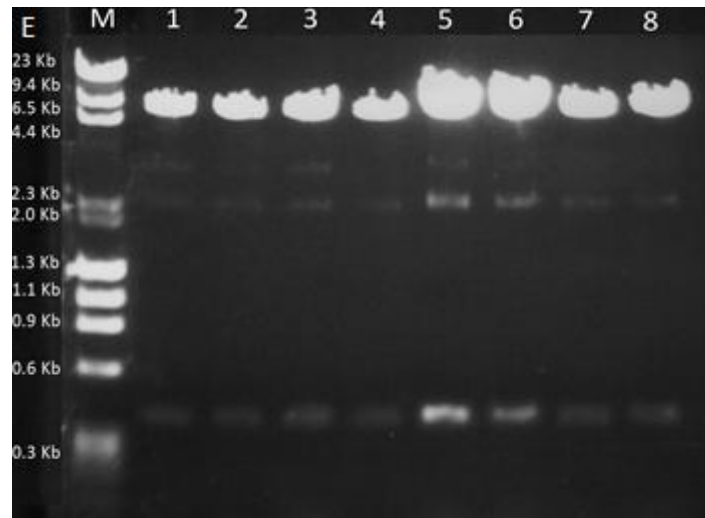


Figure 3.4 DNA digests showing PCR products excised from each of the pFUS2 constructs. Lane M in each gel represents λ HindIII/ ϕ X174 HaeIII DNA size marker. A) Lanes 1 – 12 represent *katE* clones. B) Lanes 1 – 12 represent *katG* clones. C) Lanes 1 - 6 represent *mutM* clones and lanes 7 – 12 represent *mutS* clones. D) Lanes 13 - 18 represent *mutY* clones. E) Lanes 1 – 12 represent *mutL* clones. Additional bands in E are probably undigested DNA.

3.3.2 Construction of IDM mutants in *M. loti* R7A, R7ANS and R7A Δ dct

The pFUS2 clones were confirmed by DNA sequencing and then transferred into R7A, R7ANS and R7A Δ dct via bi-parental mating (section 2.13.2) between *E. coli* S17-1 clones and the three *M. loti* strains R7A, R7ANS and R7A Δ dct. The pFUS2 clone should integrate into the gene via a single crossover and be flanked by a direct repeat of the DNA sequence that was cloned into pFUS2. Selection of the *M. loti* strains that had integrated the construct was achieved by plating out the spot matings onto G/RDM + Gm. Resultant single colonies were then single colony purified three times onto G/RDM + Gm to ensure there were no longer any *E. coli* cells present on the medium.

3.3.3 Confirmation of IDM mutants

Chromosomal DNA was extracted from 4 – 6 clones representing each putative mutant in each of the three *M. loti* background strains. The DNA was digested with an appropriate restriction endonuclease, depending on the particular construct, and the digested DNA was run overnight on 1% agarose gels. Overnight gels were checked for consistency of DNA density and then were transferred to Hybond-N⁺ nylon membranes to be used for Southern hybridisation (section 2.18). The membranes were probed with the PCR product that was amplified to create the 350-bp intragenic region. The membranes were then stripped and re-probed with labelled pFUS2 to show the presence of the vector in the mutant. The size of the expected band reflected by either probe is determined by the sum of the vector, the 350-bp PCR product and the genomic region encoding the target gene, as summarised in Table 3.. After development, the Southern hybridisations (Figure 3.5 – 3.9) were examined for the presence of bands indicating a successful mutant. Expected sizes were calculated using the *M. loti* MAFF303099 sequence.

A problem occurred when trying to confirm the *mutL* mutant. The PCR product used to create the 350-bp intragenic region failed to provide specific binding in the Southern that was performed. Subsequent attempts to obtain a new *mutL* PCR product were unsuccessful, and due to the time constraints of this study, further attempts to construct a *mutL* mutant were abandoned.

Table 3.2 Summary of expected band sizes for mutants confirmed using Southern hybridisation.

Mutant	Enzyme	Wild-type fragment size	Plus pFUS2 (7198 bp) and insert (350 bp)
<i>katE</i>	PstI	1041	8,589
<i>katG</i>	Sall	2999	10,552
<i>mutM</i>	PstI	3229	10,777
<i>mutS</i>	PstI	3420	10,968
<i>mutY</i>	Sall	2593	10,141

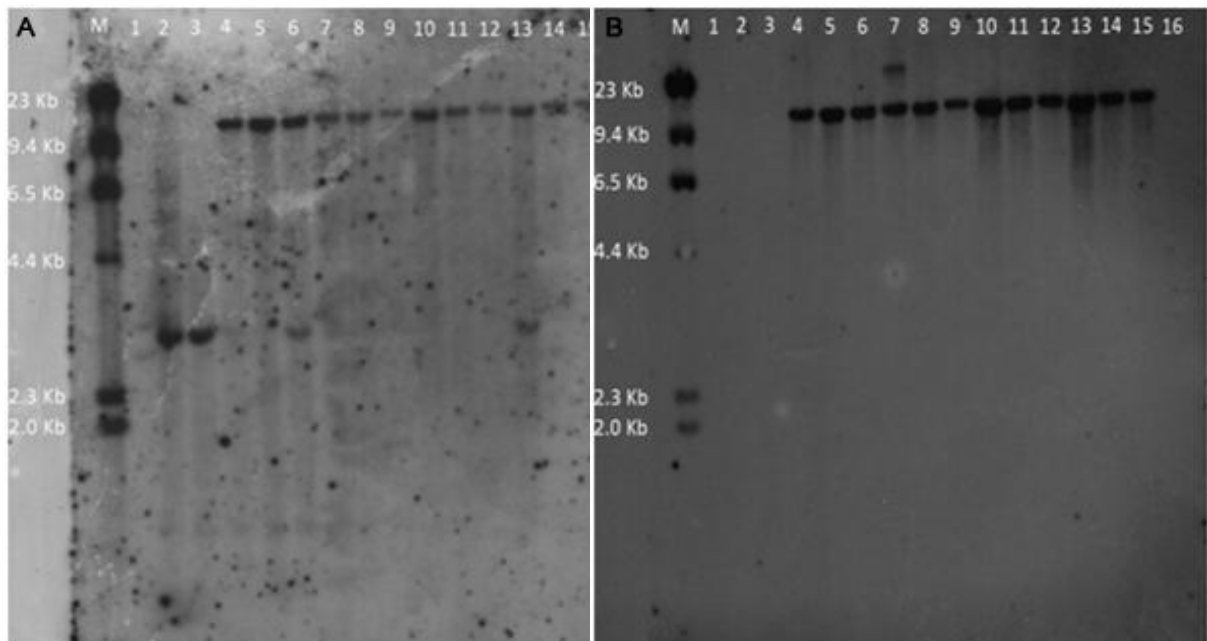


Figure 3.5 Southern hybridizations of *Sall*-digested DNA of putative *katG* mutants of R7A, R7ANS and R7AΔ*dct*. Lane M in each Southern represents λ HindIII DNA size marker. A) Shows the Southern probed with the PCR product used to create the mutant. Lane 1 is empty; Lane 2 R7A control; Lanes 3 – 6 R7A-*katG*; Lanes 7 – 10 R7ANS-*katG*; Lanes 11 – 16 R7AΔ*dct*-*katG*. B) This shows the same membrane that has been stripped and probed using labelled pFUS2. The isolates represented by lanes 5, 10 and 15 (one from each strain background) were selected.

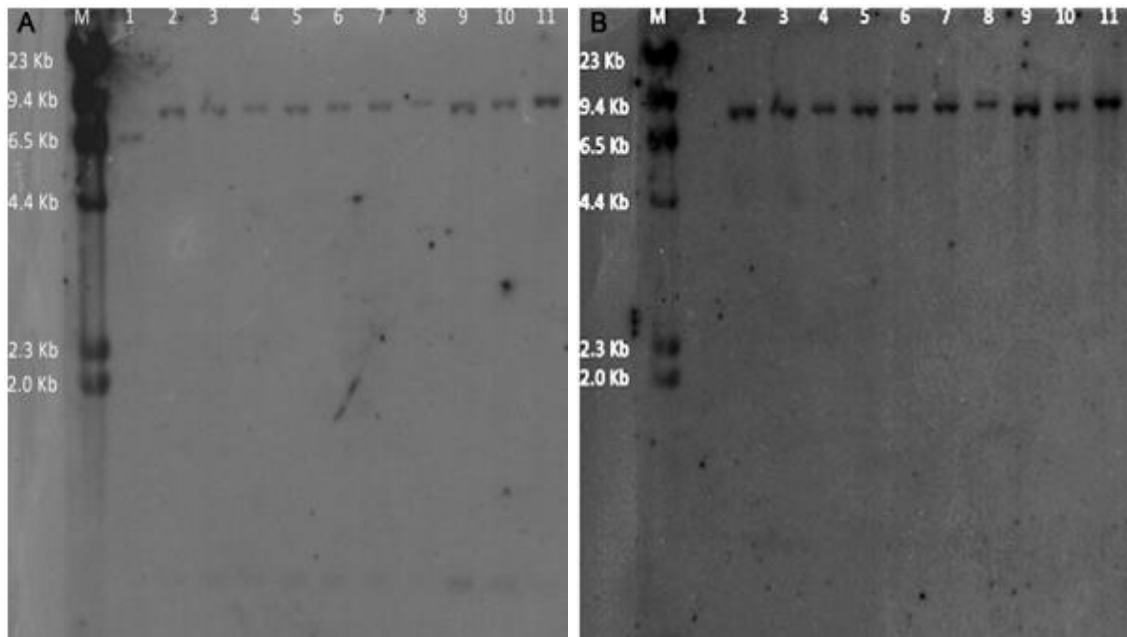


Figure 3.6 Southern hybridizations of PstI-digested DNA of putative *katE* mutants of R7A, R7ANS and R7A Δ *dct*. Lane M in each Southern represents λ HindIII DNA size marker. A) Shows the Southern probed with the PCR product used to create the mutant. Lane 1 R7A control; Lanes 2 – 5 R7A-*katE*; Lanes 6 – 8 R7ANS-*katE*; Lanes 9 – 11 R7A Δ *dct*-*katE*. B) This shows the same membrane that has been stripped and probed using labelled pFUS2. The isolates represented by lanes 2, 8 and 11 (one from each strain background) were selected.

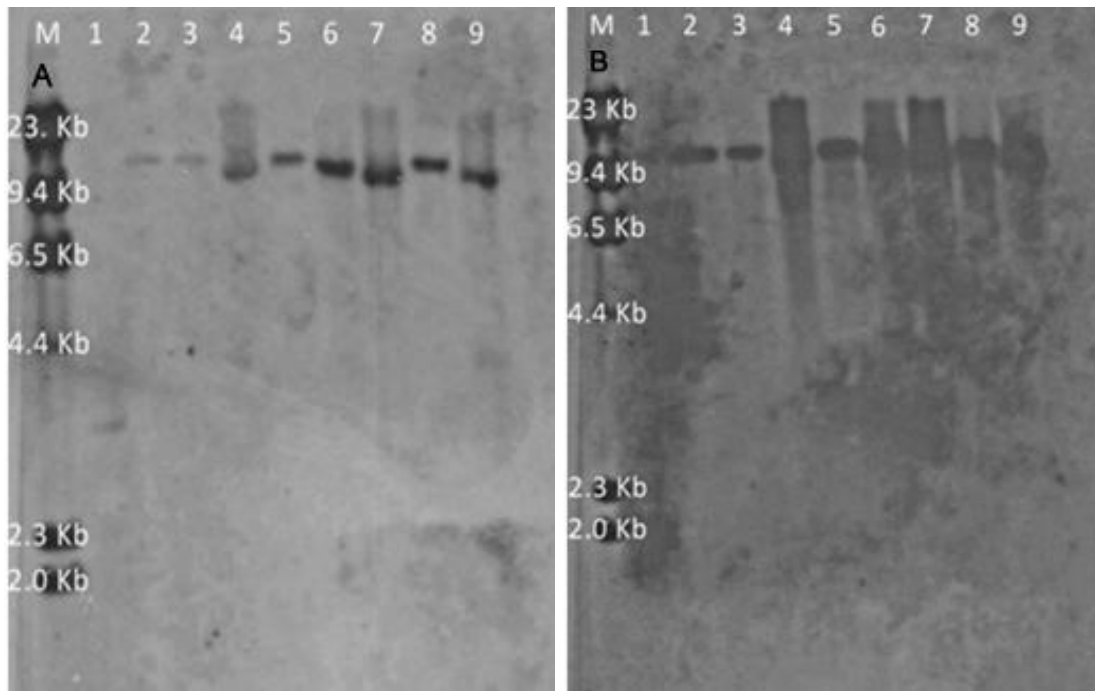


Figure 3.7 Southern hybridizations of PstI-digested DNA of putative *mutM* mutants of R7A, R7ANS and R7A Δ dct. Lane M in each Southern represents λ HindIII DNA size marker. A) Shows the Southern probed with the PCR product used to create the mutant. Lane 1 R7A control; Lane 2-4 R7A-*mutM*; Lane 5-7 R7ANS-*mutM*; Lane 8 & 9 R7A Δ dct-*mutM*. B) This shows the same Southern that has been stripped and probed using labelled pFUS2. The cultures represented by lanes 2, 5 and 8 (one from each strain background) were selected.

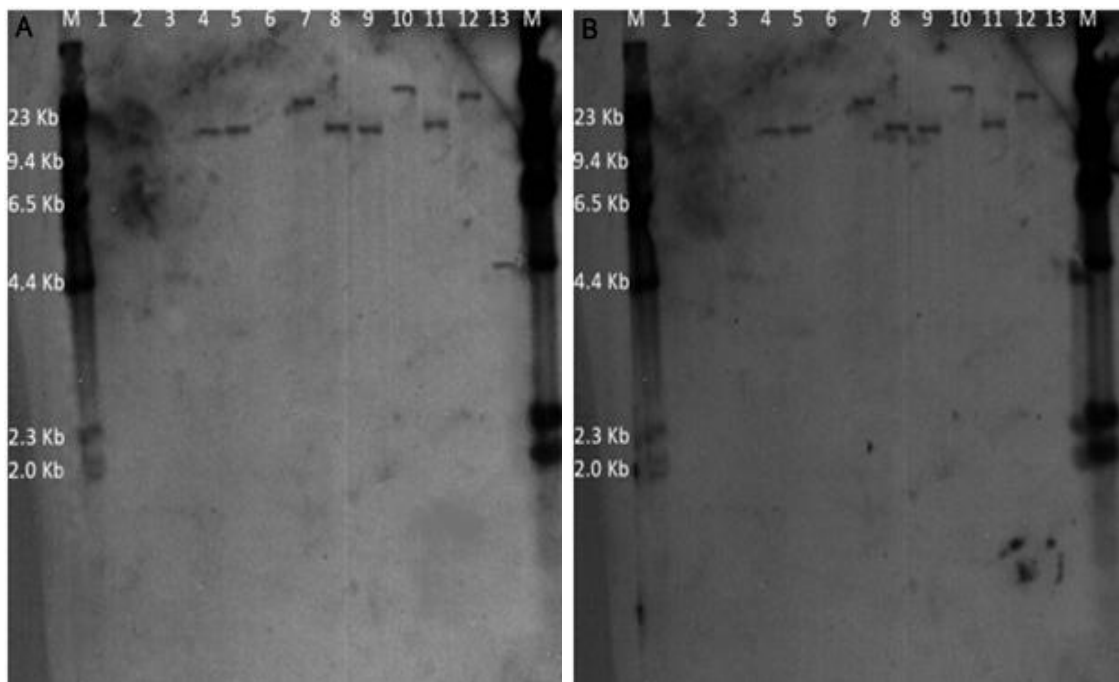


Figure 3.8 Southern hybridizations of PstI-digested DNA of putative *mutS* mutants of R7A, R7ANS and R7A Δ dct. Lane M in each Southern represents λ HindIII DNA size marker. A) Shows the Southern probed with the PCR product used to create the mutant. Lane 1 - 4 R7A-*mutS*; Lane 5- 8 R7ANS-*mutS*; Lane 9 - 12 R7A Δ dct-*mutS*. Lane 13 R7A control. B) This shows the same membrane that has been stripped and probed using labelled pFUS2. The isolates represented by lane 4, 8 and 11 (one from each strain background) were selected. Table 3.2 shows an expected wild-type band size of 3.4 kb, however lane 13 shows a band of 4.4 kb. The expected band size was originally obtained from *M. loti* MAFF303099 sequence data, however in *M. loti* R7A the fragment is 4440 bp. The difference is due to slight variation in sequence between the two strains.

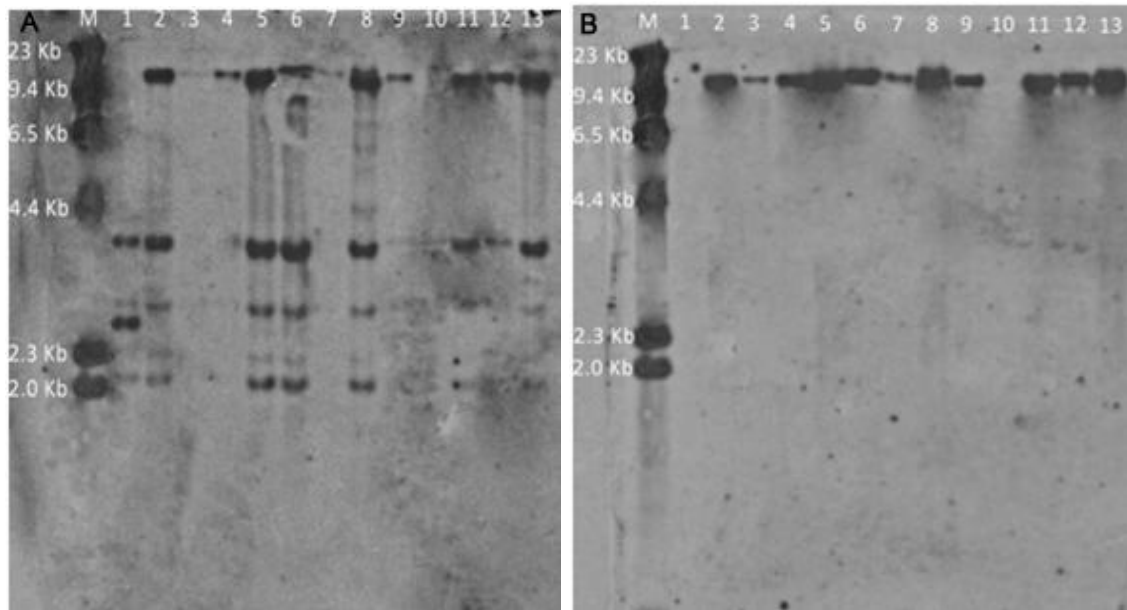


Figure 3.9 Southern hybridizations of *Sall*-digested DNA of putative *mutY* mutants of R7A, R7ANS and R7AΔ*dct*. Lane M in each Southern represents λ HindIII DNA size marker. A) Shows the Southern probed with the PCR product used to create the mutant. Lane 1 R7A control. Lane 2 - 5 R7A-*mutY*; Lane 6 - 9 R7ANS-*mutY*; Lane 10 - 13 R7AΔ*dct*-*mutY*. B) This shows the same membrane that has been stripped and probed using labelled pFUS2. The isolates represented by lane 4, 7 and 11 (one from each strain background) were selected.

3.4 *mutT* markerless deletion mutant

The *M. loti mutT* gene is only 420-bp in length and therefore is unsuitable for IDM mutagenesis. IDM mutagenesis requires a fragment of approximately 350-bp for successful homologous recombination to occur; for a gene as small as *mutT*, IDM would almost reconstitute the gene which could be enough to enable it to continue functioning. Instead a markerless deletion mutant was constructed for *mutT*. The strategy used for mutant construction is depicted in Fig. 3.10.

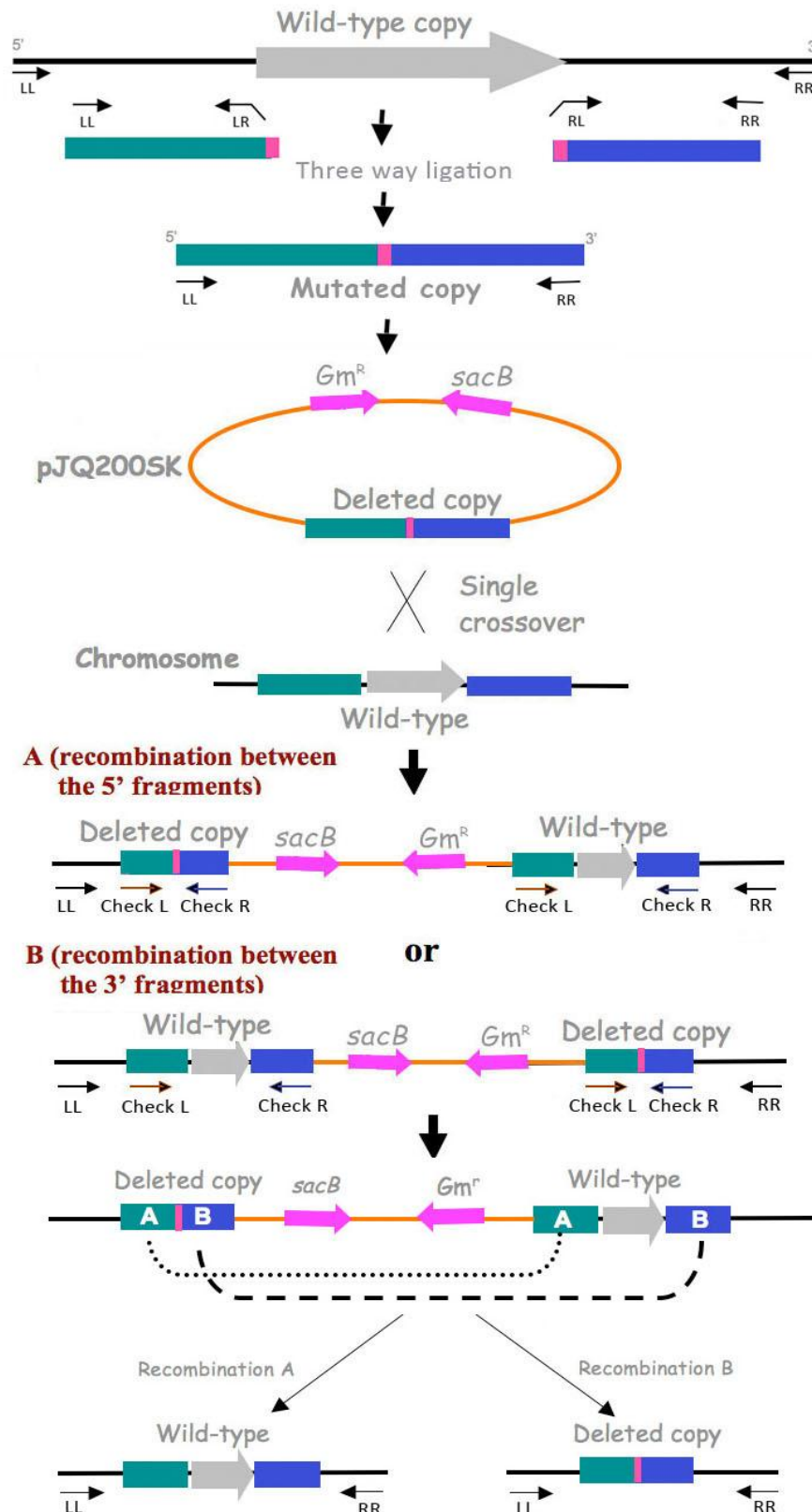


Figure 3.10 Schematic diagram of the construction of *mutT* markerless deletion mutant (Sharma, 2006).

3.4.1 Construction and confirmation of *mutT* markerless deletion mutant

Primers MutTpJQLL, MutTpJQLR, MutTpJQRL and MutTpJQRR were designed to amplify two regions flanking the *mutT* gene. This resulted in a left arm and a right arm either side of the gene, plus approximately 100-bp of the gene itself. Restriction sites were incorporated within the ends of the primers to facilitate cloning. A PCR product containing a short region at the start of *mutT* and the preceding 1200-bp was amplified using primers MutTpJQLL and MutTpJQLR, purified and then digested with *SpeI* and *HindIII*. A second 1200-bp PCR product containing the stop codon of *mutT* and the following 1200-bp of sequence was amplified using primers MutTpJQRL and MutTpJQRR and then digested with *XhoI* and *HindIII*. The two PCR products were then examined by agarose gel electrophoresis to confirm they were the correct size (Figure 3.11).

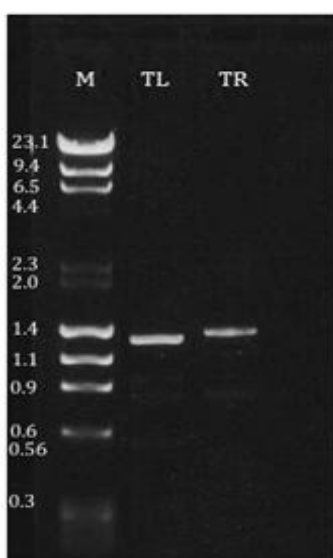


Figure 3.11 Confirmation of *mutT* PCR products. Lane M represents λ *HindIII*/ ϕ X174 *HaeIII* marker. The sizes of the marker bands are shown in kb. Lane TL represents the left arm PCR product produced using primers MutTpJQLL, MutTpJQLR, Lane TR represents the right arm.

The 2 arms were then purified, combined and ligated into the suicide vector pJQ200SK (Quandt and Hynes 1993) which had been digested with *XhoI* and *SpeI* and treated with alkaline phosphatase. The ligation was transformed into *E. coli* S17-1 by electroporation and plated onto LB (Gm) agar. Plasmid DNA was prepared from transformants and digested with *SpeI* and *XhoI* to locate a clone with a 2.4-kb insert consisting of the two 1.2-kb regions that flank the gene fused at the *HindIII* site, with a short truncated version of the gene located in the centre. Digestion with *SpeI* and *XhoI* should result an insert band of approximately of 2.4-kb plus a second band of approximately 5.3-kb representing the vector (Figure 3.12).

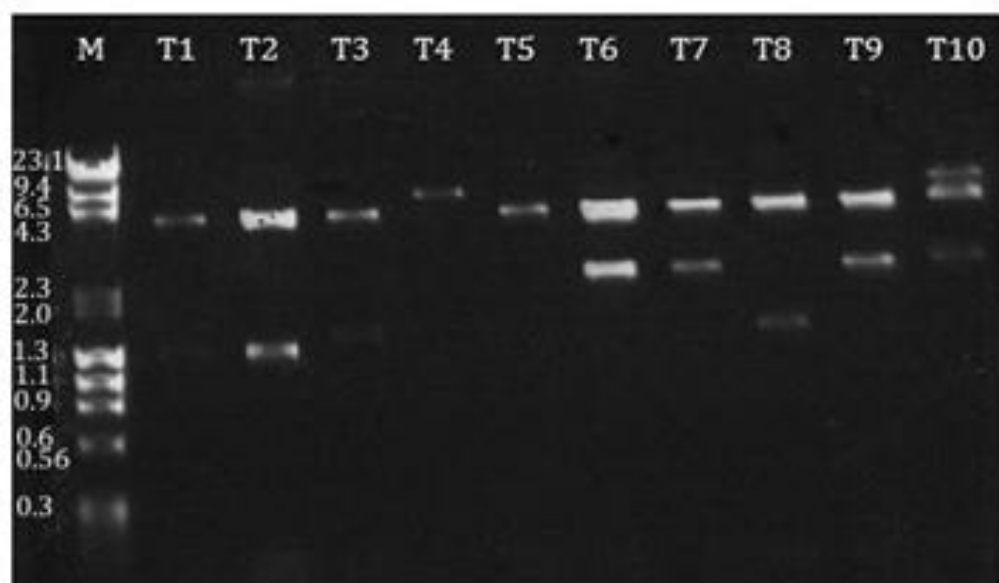


Figure 3.12 Confirmation of the *mutT* pJQ200SK clone. Lane M represents λ HindIII/ ϕ X174 HaeIII marker. The sizes of the marker bands are shown in kb. Lanes T1 through to T10 represent a series of pJQ200SK clones that were digested with XhoI and SpeI. Digestion with these endonucleases is expected to result in a 5.3-kb vector band and the 2.4-kb insert band. T6, T7, T9 and T10 showed bands of expected sizes.

Two clones designated T6 and T7 were selected for confirmation by DNA sequencing, performed using the universal forward and reverse sequencing primers and two custom primers designated MutTseqcheckrev1 and MutTseqcheckfwd1. A single confirmed plasmid clone designated pT6 was chosen to construct the mutants. Biparental matings were performed to transfer the plasmid from *E. coli* S17-1 to the *M. loti* strains R7A, R7ANS and R7A Δ dct. Transconjugants were passaged on G/RDM plates containing gentamicin three times. Colonies were screened by PCR to identify strains in which the plasmid had inserted via a single cross-over resulting in a strain containing both the wild-type and deletion variant *mutT* sequences separated by the plasmid. *M. loti* DNA was prepared from 3-day TY broth cultures using the PrepMan[™] Ultra reagent, and subjected to PCR using primers MutTpJQcheckshortL and MutTpJQcheckshortR which flank *mutT* in R7A. The amplification of two PCR products of the anticipated sizes representing the wild-type *mutT* gene and the deletion variant indicated that the desired single crossover recombination event had occurred (Figure 3.13).



Figure 3.13 Selection of *M. loti* isolates in which the T6 pJQ200SK clone has integrated via a single crossover. Lane M represents λ HindIII/ ϕ X174 HaeIII marker. The sizes of the marker bands are shown in kb. Lane 1 represents a negative control PCR reaction which contained no template DNA. Lane 2 represents a positive control which was run using *M. loti* R7A template DNA. Lanes T1 through to T12 represent different gentamicin-resistant clones containing the *mutT* pJQ200SK construct. Successful clones should show two bands, as is the case with T1, T3, T5, T6, T7, T11 and T12, a wild-type band of 910-bp and a 600-bp band representing the deletion variant. T10 was left out due to contamination of the sample. Clones T1 – T4 were in the R7A background, T5 – T8 were in the R7ANS background and T9 – T12 were in the R7A Δ dct background.

The final stage of constructing the markerless deletion mutants involved using a counterselection technique to select cells which had lost pJQ200SK as a result of homologous recombination between the duplicated sequences present in the strain containing the integrated vector. There are two possibilities for the second crossover event: the original crossover event will be reversed which will remove the vector and the mutated gene meaning the chromosome will return to its original wild-type form or the recombination will remove the wild-type gene leaving behind the mutant gene with the markerless deletion. The pJQ200SK vector contains the *sacB* gene which produces lethal metabolites in the presence of 5% sucrose. Two colonies from each of the strains designated T1, T3, T5, T7, T11 and T12 (Figure 3.13) were used to inoculate TY broths which were grown to stationary phase. Serial dilutions of these broths were then plated on RDM plates containing 5% sucrose. Single colonies taken from plates inoculated with dilutions of 10^{-3} and 10^{-4} were colony-purified on G/RDM with no antibiotics. Patch plating was performed to find gentamicin-sensitive sucrose-resistant strains which had presumably lost pJQ200SK as a result of homologous recombination between the duplicated sequences.

Genomic DNA from the strains that successfully grew on sucrose and were gentamicin-sensitive was extracted and used as template DNA for a subsequent PCR. The final confirmation of the markerless deletion *mutT* mutant was performed by PCR using the MutTpJQcheckshortL and MutTpJQcheckshortR primer pair that flank the deletion. The results from this PCR are shown in Figure 3.14

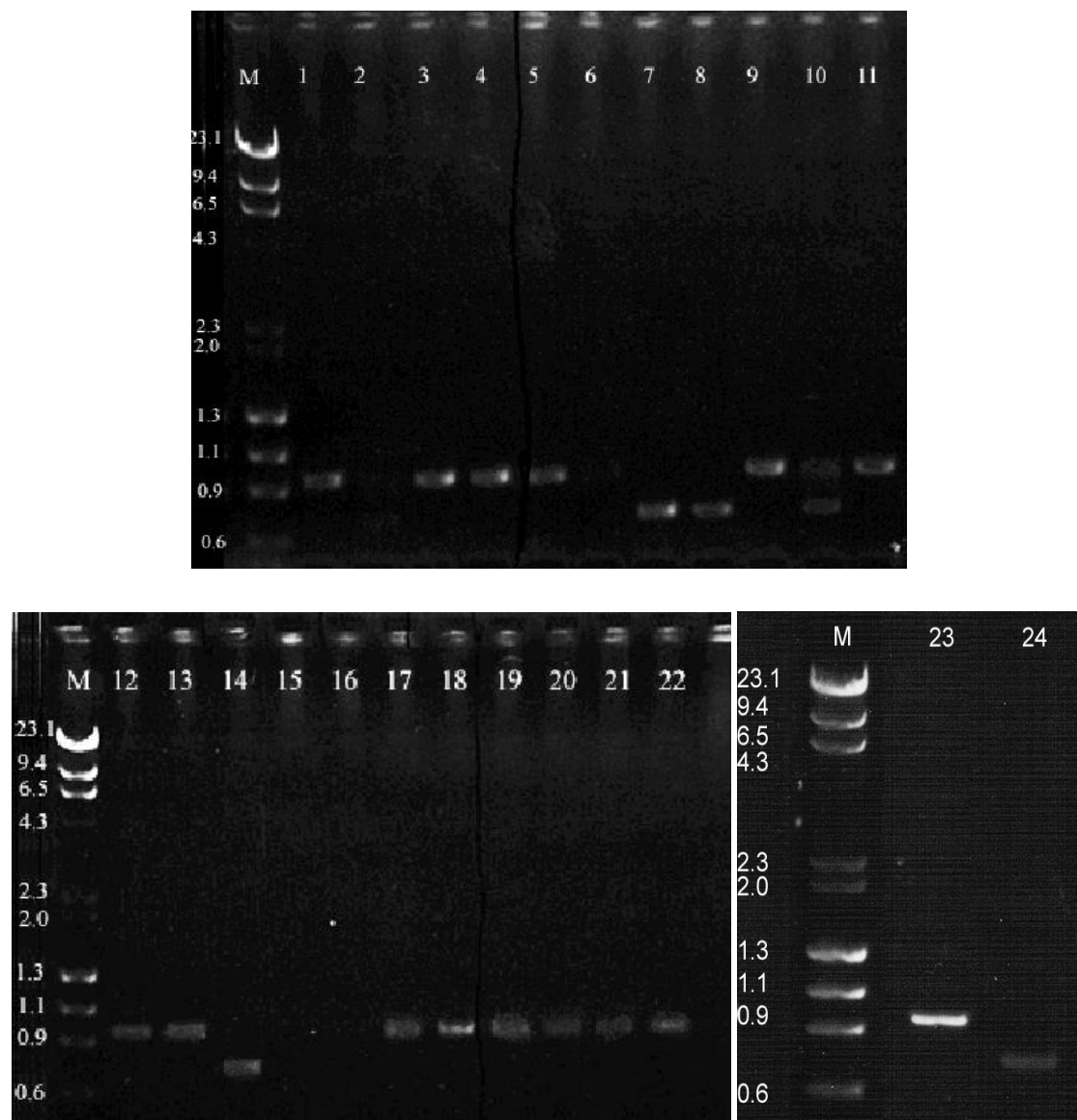


Figure 3.14 Confirmation of the *mutT* mutants. This gel shows the presence of either a wild-type band of 910-bp or a 600-bp band representing the deletion variant. Lanes labelled M represent λ hindIII/ ϕ X174 HaeIII marker. The sizes of the marker bands are shown in kb. There are three different background strains present for these *mutT* clones. Lanes 1 - 8 are in *M. loti* R7A, Lanes 9 – 16 are in *M. loti* R7ANS and lanes 17 – 24 are in *M. loti* R7A Δ dct. The isolates selected were 8, 14, and 24.

3.5 Phenotypic characterisation of catalase mutants

3.5.1 The mutator phenotype of *M. loti* R7A Δ dct catalase mutants

The mutator phenotype of the *M. loti* R7A Δ dct-*katG* and R7A Δ dct Δ *katE* mutants was tested by observing the frequency of appearance of Dct⁺ colonies by spread plating onto succinate-containing media. This was carried out in parallel; identical plates were prepared differing only in their NA concentration, 1 μ g/ml and 50 μ g/ml. The plates were incubated for 2 weeks and the cumulative appearance of colonies was recorded. The results showed a clear increase in the number of Dct⁺ revertants in strain R7A Δ dct-*katG* compared with the control strain, R7A Δ dct (Figure 3.15A). In both strains, the Dct⁺ colonies began appearing after 7 or 8 days incubation, whereas the Dct⁺ phenotype of wild-type R7A colonies was clearly discernible by 5 days incubation. Hence for both strains, the Dct⁺ mutants were not pre-existing in the culture but only arose after extended culture on succinate media. There appeared to be a negative impact on mutation rate of NA in excess (50 μ g/ml), which is consistent with previous work (Ashbrook 2012). These results suggested that strain *M. loti* R7A Δ dct-*katG* underwent an increased mutation rate compared with the control strain whilst under starvation stress. Given that this particular mutant strain was missing *katG* (bifunctional catalase/peroxidase) which is responsible for the degradation of H₂O₂ into H₂O and O₂, this result implicates oxidative stress in the generation of the mutants.

The *katE* mutant presented a different effect to that seen in the *katG* mutant. Comparison of the number of colonies that arose in strains R7A Δ dct-*katE*, and R7A Δ dct revealed a decrease in the number of mutants that arose in the mutant strain compared with the wild-type (Figure 3.15B). There appeared to be a significant decrease in the number of mutants arising in the *katE* mutant strain, however the slope of the line is similar between the mutant and the wild-type and a slightly later onset may account for the apparent difference between the strains.

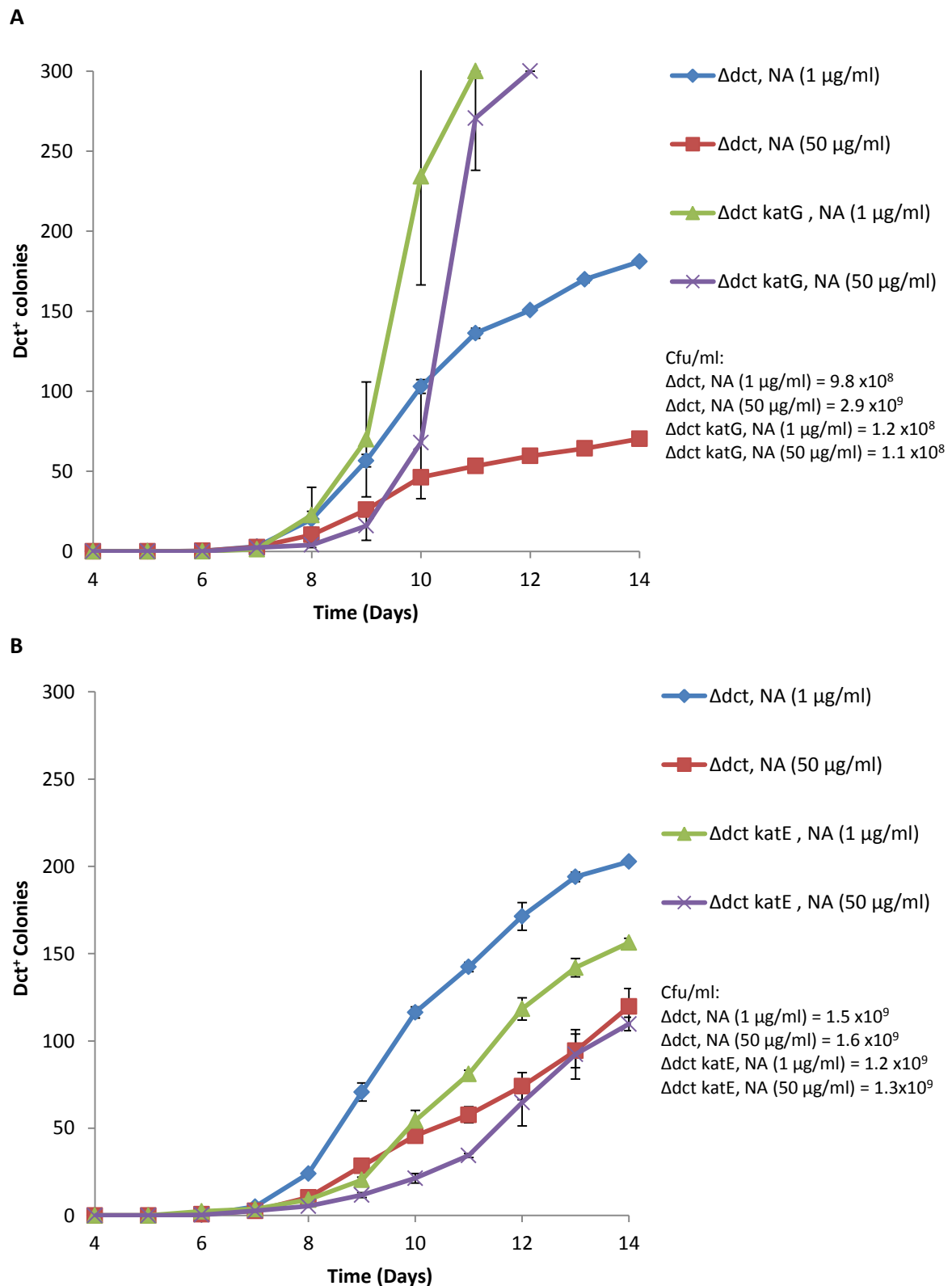


Figure 3.15 The cumulative appearance of Dct⁺ revertant colonies grown on succinate over time for catalase mutants in the *M. loti* R7AΔdct background plated at the 1×10^{-3} dilution. A) Appearance of Dct⁺ colonies in *M. loti* R7AΔdct-katG and *M. loti* R7AΔdct. B) Appearance of Dct⁺ colonies in *M. loti* R7AΔdct-katE and *M. loti* R7AΔdct. Each strain was grown in the

presence of two different concentrations of NA (1 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$) and the cfu/ml for each strain is displayed on the figure, calculated by counting microcolonies on plates at the 10^{-6} dilution. Error bars shown at each time point for this, and all subsequent graphs, represent the standard error calculated using plate counts obtained from three independent experiments. Note that some error bars are too small to be seen.

3.5.2 Mutator phenotype of *katG* mutation in the *M. loti* R7ANS

background

The *M. loti* R7ANS-*katG* mutant was tested by observing the frequency of appearance of Dct⁺ colonies by spread plating onto succinate media, as described above in section 3.5.1. The effect of NA on this strain was substantially different than that observed in the Δdct background. The R7ANS strain contains no NAD locus and without the presence of these genes, or a high level of NA, no revertant colonies arise. Overall, the *katG* mutant strain showed an increase in the number of Dct⁺ colonies in the presence of NA at 1 $\mu\text{g/ml}$ and at 50 $\mu\text{g/ml}$ when compared with the R7ANS control strain (Figure 3.16). Unlike the Δdct background, the results in the NS background showed an increase in the mutator phenotype on media containing an excess of NA (50 $\mu\text{g/ml}$) compared with media containing 1 $\mu\text{g/ml}$, which reflects observations made previously (Ashbrook 2012). The *katG* mutant showed a significant mutator phenotype, which was further intensified with addition of 50 $\mu\text{g/ml}$ NA. These results suggest that not only is oxidative stress the likely cause of the effect, but NAD also plays a critical role in modulating the effect of the mutator phenotype observed.

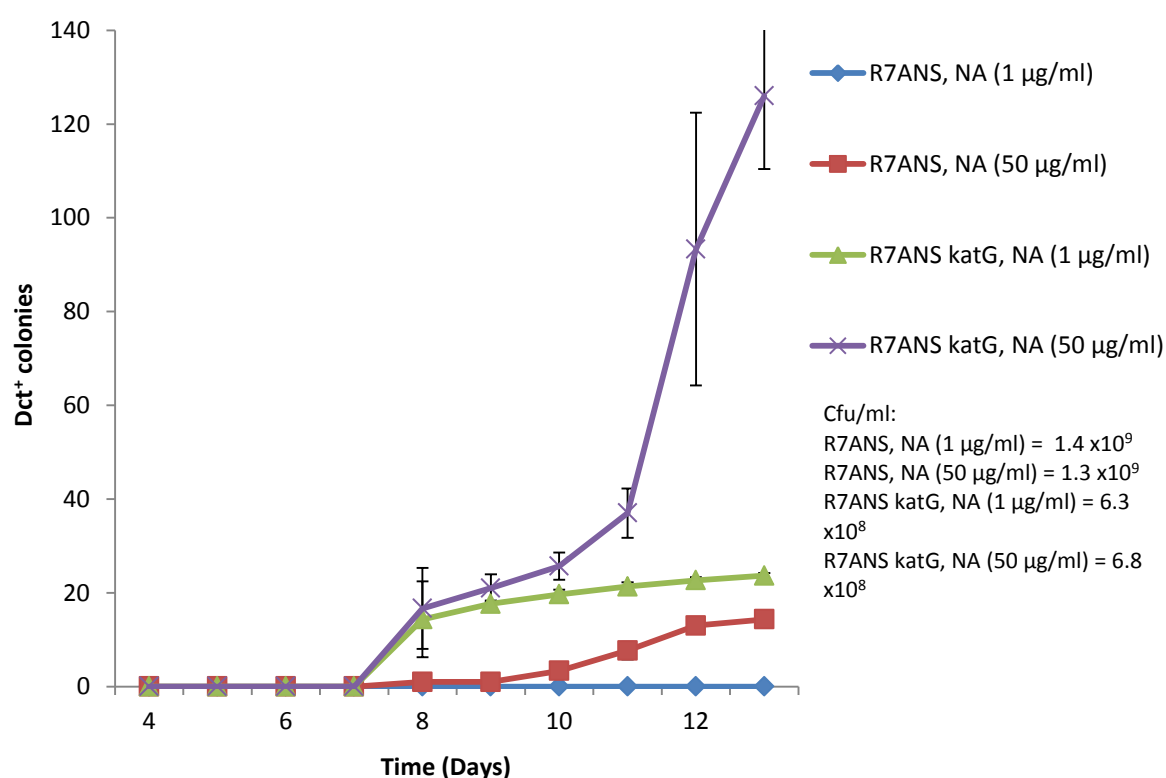


Figure 3.16 Appearance of Dct⁺ colonies in *M. loti* R7ANS-*katG* and *M. loti* R7ANS over time, performed in the same manner as detailed in Fig 3.15.

3.5.3 Appearance of antibiotic resistance colonies on rifampicin with the *katG* R7ANS mutant strain

In this experiment strains R7ANS-*katG* and R7ANS were plated onto G/RDM containing 200 µg/ml of rifampicin (Figure 3.17). An initial screen was performed to determine the most suitable concentration to add to the media (data not shown). The results show only a small difference in the number of resistant colonies arising. This is expected as rifampicin is considered bactericidal; as soon as the cells were placed on this medium their DNA-dependent RNA polymerase is inhibited and therefore RNA synthesis is inhibited (Campbell, Korzheva *et al.* 2001). The level of NA should show no effect on the cells in this scenario because the cells are being killed before they can reach an NADH/NADPH imbalance. The small increase in resistant colonies observed in the *katG* mutant strains could be due to the internal production of H₂O₂ that occurs as an inevitable outcome of aerobic metabolism. After day five very few new colonies arose, indicating that the increase in mutation that was observed for the *katG*-deficient strains would have occurred pre-plating. Although these are NS strains, there appeared to be no difference conferred by the varying concentrations of NA; especially when compared with the variation observed in the experiment detailed previously (Figure 3.16).

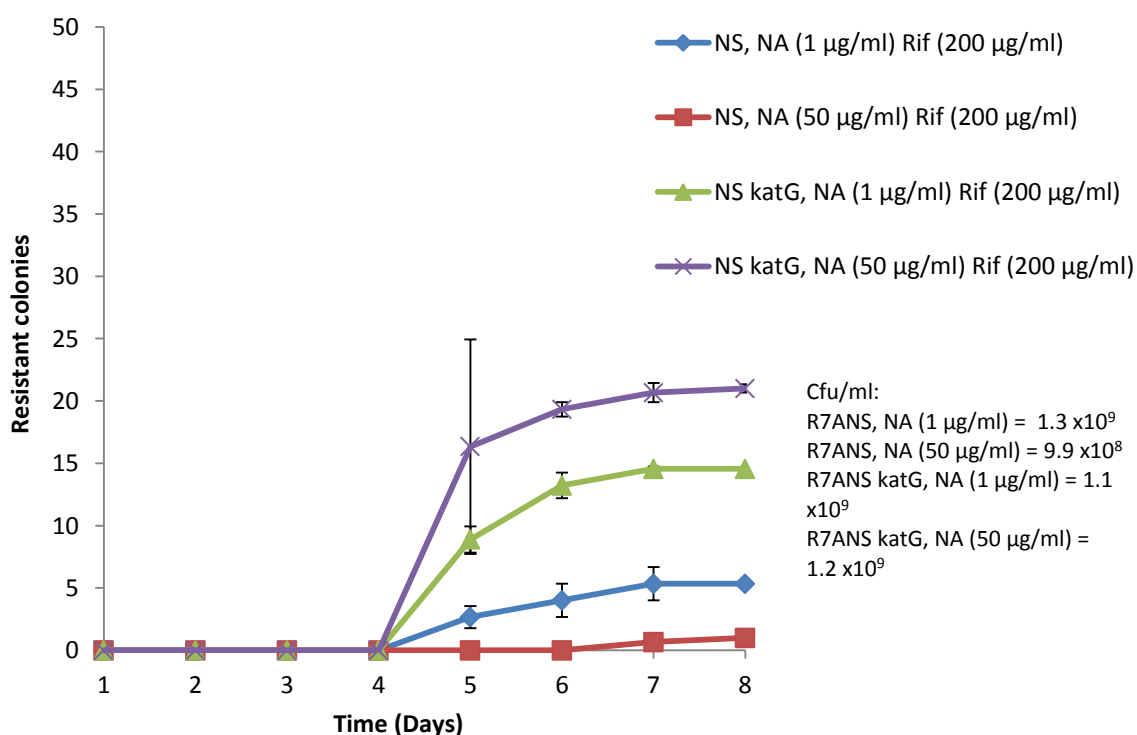


Figure 3.17 Appearance of rifampicin resistant colonies of *M. loti* R7ANS-*katG* and *M. loti* R7ANS over time. The cumulative appearance of rifampicin resistant colonies grown on G/RDM containing rifampicin (200 µg/ml) at two different concentrations of NA (1 µg/ml and 50

µg/ml). The cultures were standardised to an OD₆₀₀ of 1, and 100 µl was plated. There appears to be a small but still significant effect conferred by the *katG* mutation.

3.5.4 Hydrogen peroxide sensitivity of *katG* mutants

To assess the effects of externally added H₂O₂ on the catalase mutants, an inhibition zone assay adapted from (Hanyu, Fujimoto *et al.* 2009) was carried out. Hanyu *et al.* showed that their *katG* mutant had a significant increase in sensitivity to exogenous H₂O₂ compared to the wild-type *M. loti* MAFF303099.

Cultures were grown overnight in triplicate and approximately 1.0×10^8 cells were mixed with 10 ml 0.7% TY agar overlays. Once the cells were added, the overlay was poured onto TY agar plates containing Gm. Six-mm Whatman filter paper discs containing seven µl of 0.61 M H₂O₂ were added to the centre of the plate, and the plates were then incubated at 28°C for 4 days. The diameter of the clear zone surrounding the disc for each strain was recorded in mm. The effect of the *katG* mutation was analysed in the three different *M. loti* backgrounds, with complemented strains included. *M. loti* MAFF303099 was also included as a reference because it was the strain used in the study by Hanyu *et al.* The concentration of H₂O₂ used for this experiment was chosen after performing a preliminary assay in which the organism was exposed to a two-fold serial dilution of H₂O₂ to assess which concentration would provide suitable zones of inhibition for measurement (data not shown).

The results from the assay showed a significant increase in sensitivity to exogenous H₂O₂ in the three *katG* mutants tested in comparison to the wild-type strains (Figure 3.18). The complemented strains contained a wild-type copy of *katG* on plasmid pFAJ1708. These strains restored the wild-type phenotype providing increased tolerance to H₂O₂. In each case, complementation decreased the zone of inhibition to less than that of the parental strains. This is likely due to multiple copies of the plasmid containing *katG*. These data confirm the results of Hanyu *et al.*, that *katG* has an important role in the protection of the cell to oxidative stress caused by H₂O₂, and provide further evidence for oxidative stress as the mechanism underlying the NAD-dependent mutator phenotype.

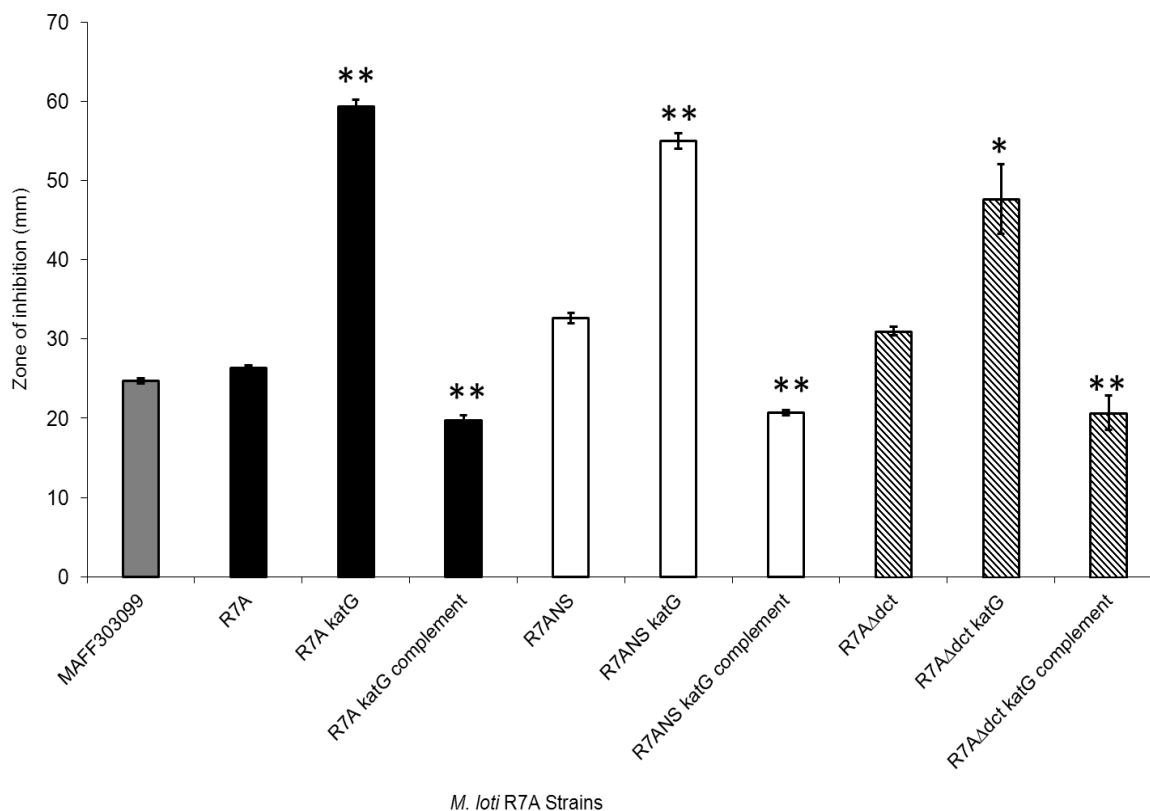


Figure 3.18 H₂O₂ zone of inhibition assay. Each bar represents the average diameter from triplicate assays of the zone of inhibition formed around a disc containing 0.61 M H₂O₂ for each of the strains listed in the figure. Four *M. loti* backgrounds are represented: MAFF303099 (grey), R7A (black), R7ANS (white), and R7AΔ*dct* (diagonal lines). With the exception of MAFF303099, each strain is represented along with their *katG* mutant and complemented counterparts. The p-value above some bars indicated by asterisks was calculated using an unpaired two-sample t-test. The asterisks (*P < 0.05, ** P < 0.01) represent the statistical difference between the data point it is above and the previous data point. A p-value below 0.05 denotes that the difference between the two means is statistically significant.

3.5.5 Viability of catalase mutants in TY broth

A series of experiments involving the catalase mutants relied on accurate OD₆₀₀ readings using a spectrophotometer. Of concern was the fitness of mutant cells in TY broth over time. To ensure that the mutant cell population in broths measured using spectroscopy was composed of viable cells, a viability curve was carried out for these mutants. The catalase mutants were inoculated as a 1 in 50 dilution from stationary phase 5 ml TY seeder broths into 100 ml TY broths which were grown for 150 h. Samples were spread onto G/RDM 3 times daily, which allowed for calculation of the cfu/ml. The experiment was carried out in triplicate for each strain, with three plates spread for each dilution plated. The curve was carried out in TY medium as opposed to RDM because rhizobia do not tend to survive as well in TY (John Sullivan, personal communication); therefore any observable viability deficiency would be expected to be exacerbated in this medium.

Growth curves were created using the OD₆₀₀ readings and the cfu/ml counts which were obtained at each sample point (Figure 3.19). Analysis of the curves concluded that there was no significant reduction in the viability of either the *R7AΔdct-katE* or *R7AΔdct-katG* mutant in comparison to the wild-type *R7AΔdct* in TY liquid medium. The number of catalase mutant cells predicted using an OD₆₀₀ reading should therefore be an accurate representation of the number of viable cells, and will not be skewed by the presence of cells that have died off early.

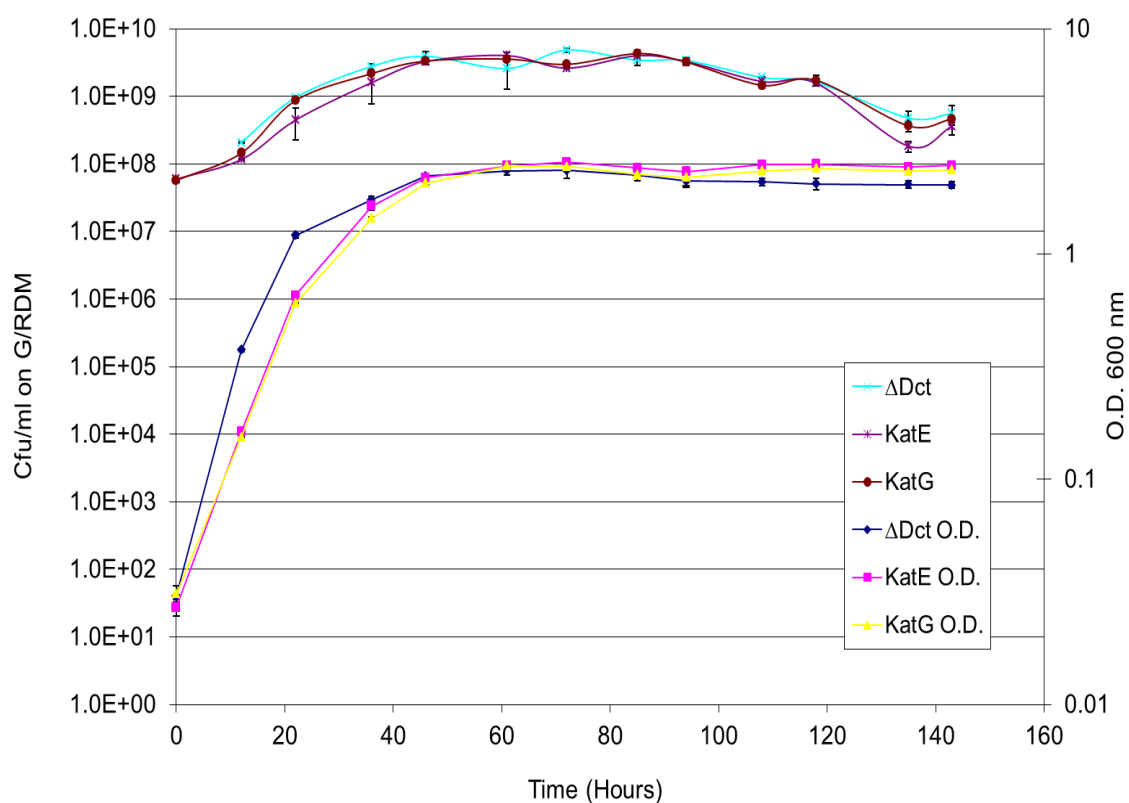
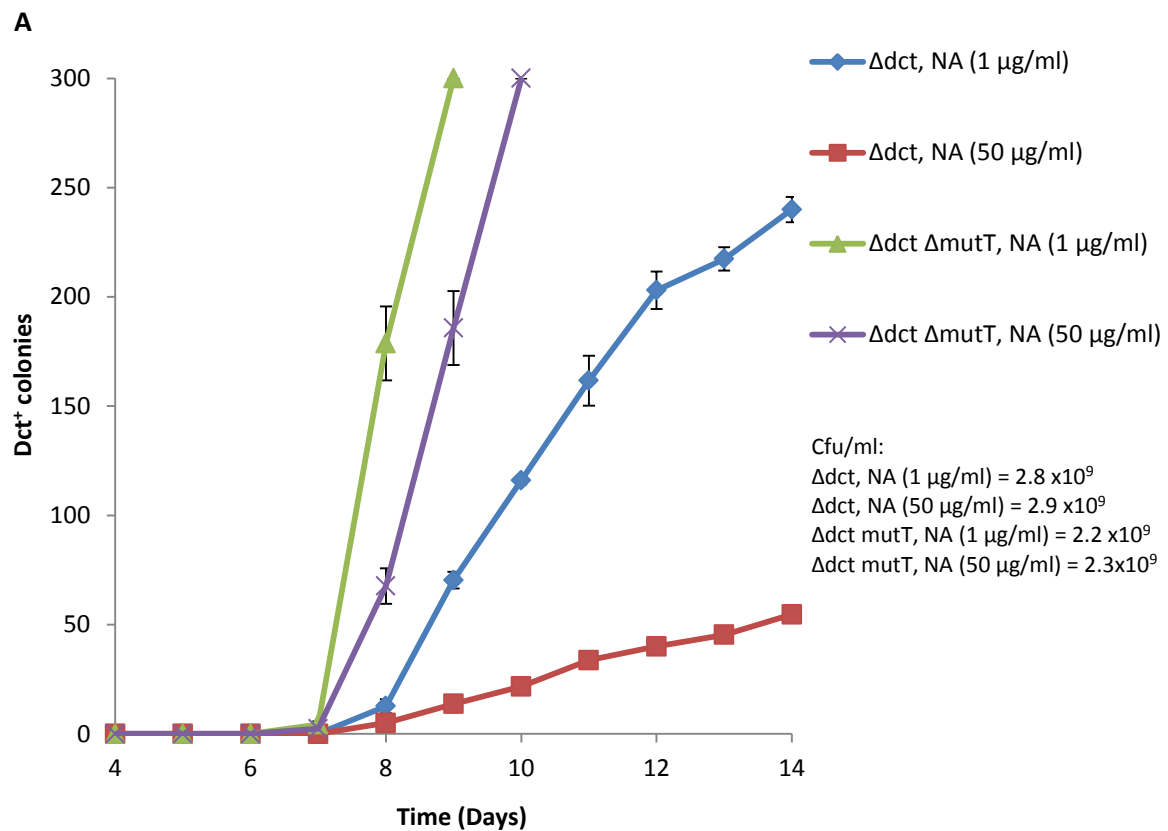


Figure 3.19 Viability curve of *M. loti* R7A Δ dct-katE, *M. loti* R7A Δ dct-katG and *M. loti* R7A Δ dct. Triplicate 100 ml TY broths were inoculated with 2 ml of stationary phase R7A culture. Broths were grown for 150 hours at 28°C with shaking at ~160 rpm. The broths were sampled at the indicated time points with the corresponding OD₆₀₀ and cfu/ml determined.

3.6 GO repair system mutants

3.6.1 Mutator phenotype of GO repair system mutants of *M. loti* R7A Δ dct

The *M. loti* R7A Δ dct-*mutY*, R7A Δ dct-*mutM* and R7A Δ dct Δ *mutT* mutants were tested by observing the frequency of appearance of Dct⁺ colonies by spread plating onto succinate containing media in the same way as detailed in section 3.5.1. The results showed a clear increase in the number of Dct⁺ revertants in the strains R7A Δ dct Δ *mutT* and R7A Δ dct-*mutY* compared with the control strain, R7A Δ dct (Figure 3.20A & 3.20B). These results show that these mutants underwent a significantly higher mutation rate than the control strain. However the mutant strain R7A Δ dct-*mutM* showed only a small difference in Dct⁺ mutant appearance compared to the control strain at 1 μ g/ml NA, and no difference at 50 μ g/ml NA (Figure 3.20C), indicating the *mutM* mutation had no effect on the mutation rate in this experiment.



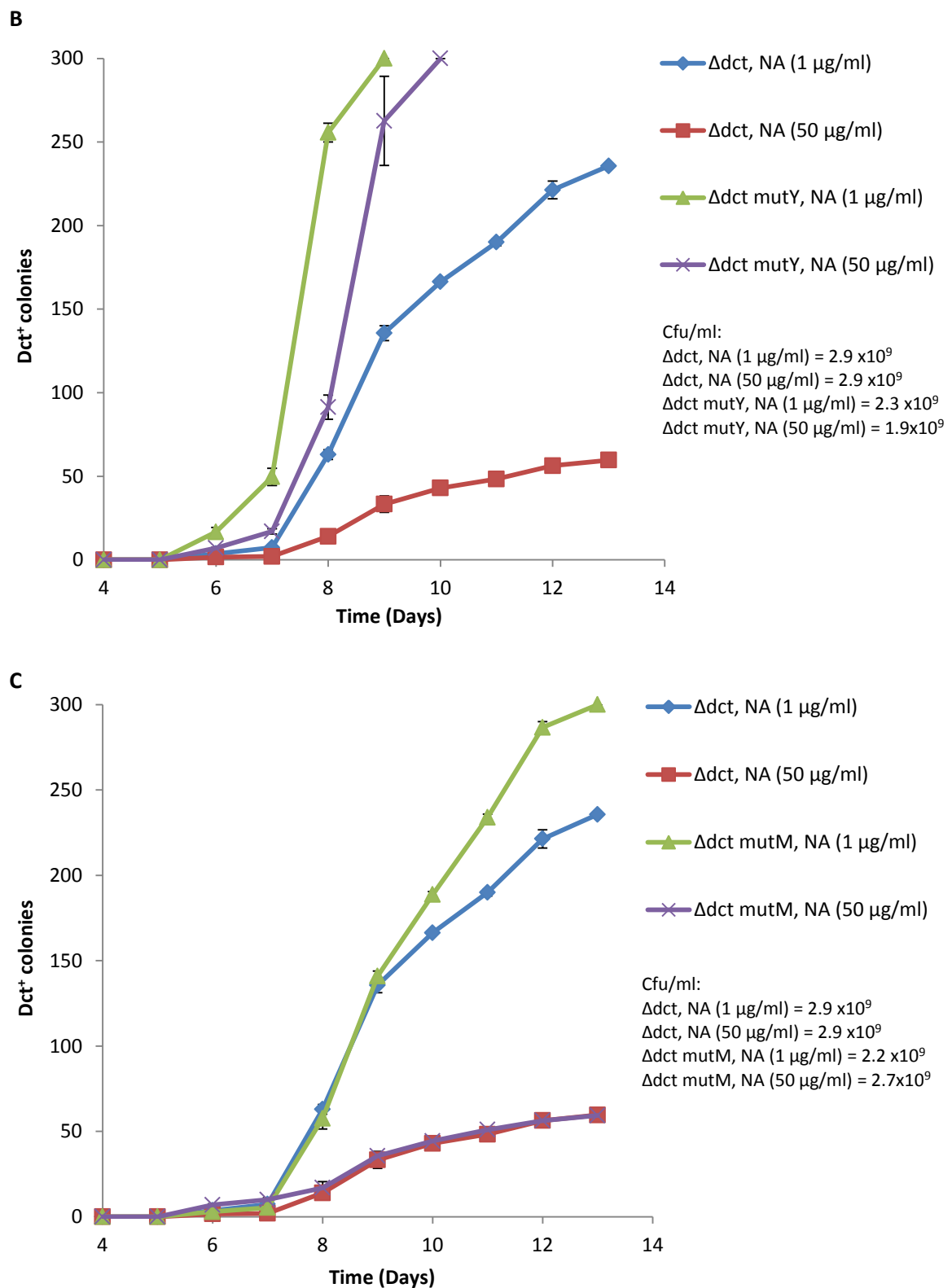


Figure 3.20 The cumulative appearance of Dct⁺ revertant colonies grown on succinate over time for mutants of the GO repair system constructed in the *M. loti* R7AΔdct background. The results are presented in the same manner as is detailed in Figure 3.15. A) Appearance of Dct⁺ colonies in *M. loti* R7AΔdctΔmutT and *M. loti* R7AΔdct. B) Appearance of Dct⁺ colonies in *M.*

loti R7A Δ dct-*mutY* and *M. loti* R7A Δ dct. C) Appearance of Dct⁺ colonies in *M. loti* R7A Δ dct-*mutM* and *M. loti* R7A Δ dct.

3.6.2 Mutator phenotype of GO repair system mutant of *M. loti* R7ANS

The effect of the *mutT* mutation in the R7ANS background was assayed using the same method as the previous experiment (section 3.6.1). At 1 µg/ml of NA, R7ANS showed no Dct⁺ revertant colonies over time, reiterating the requirement for the *nadABC* locus or excess NA to achieve a mutator phenotype. R7ANS in the presence of 50 µg/ml NA produced revertants as expected and the introduction of the *mutT* mutation had a significant effect on the number of Dct⁺ colonies that appeared. A few Dct⁺ revertants were observed in the R7ANS *mutT* mutant at 1 µg/ml NA whereas such mutants only occurred in R7ANS in the presence of excess NA (Figure 3.21). The finding that the combination of the two substantially increased the number of revertant colonies provides further evidence that the mutator phenotype induced by excess NA is caused by increased oxidative stress.

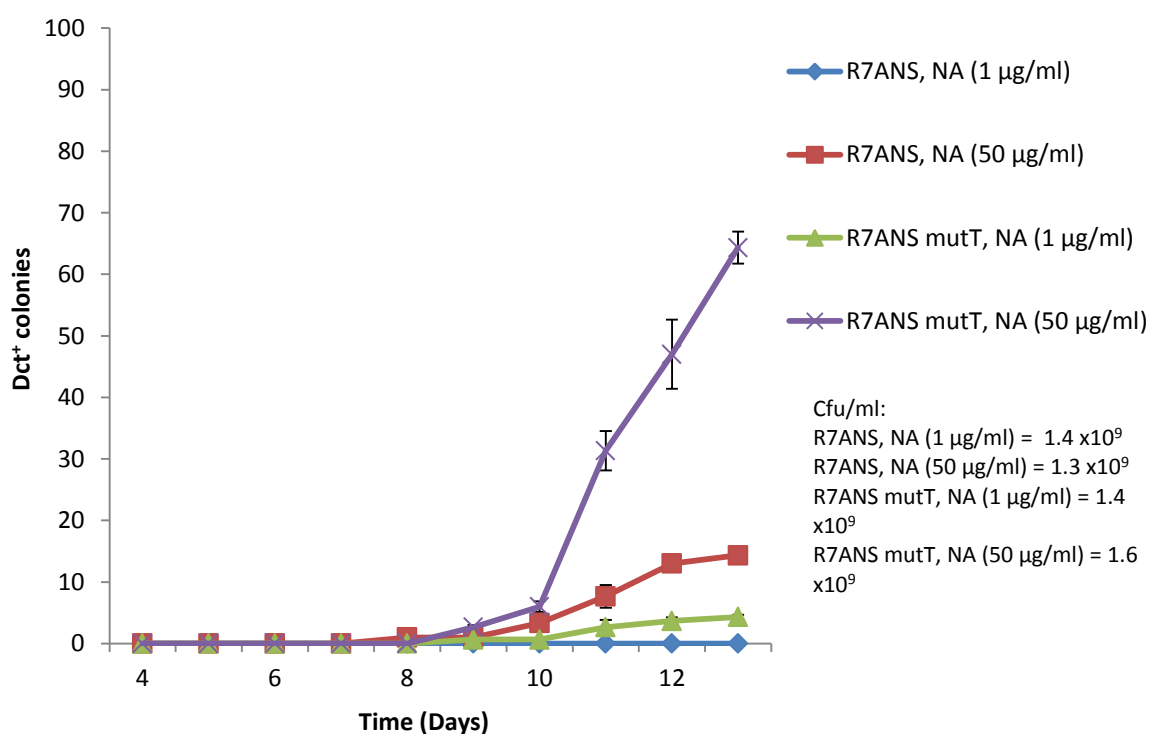


Figure 3.21 The cumulative appearance of Dct⁺ colonies in *M. loti* R7ANSΔ*mutT* and *M. loti* R7ANS over time. The R7ANSΔ*mutT* mutant strain shows a significant increase in the number of Dct⁺ colonies, showing a higher mutation rate in this strain.

3.6.3 Appearance of antibiotic resistance colonies on rifampicin with the *mutT* R7ANS mutant strain

In this experiment strains *M. loti* R7ANS Δ *mutT* and *M. loti* R7ANS were plated onto G/RDM containing 200 μ g/ml rifampicin (Figure 3.22). The results showed a difference of only a few colonies, consistent with results found for the *katG* mutant described in Section 3.5.3. This result again suggests that oxidative stress is not induced in cells grown in G/RDM and that are therefore not under carbon starvation stress.

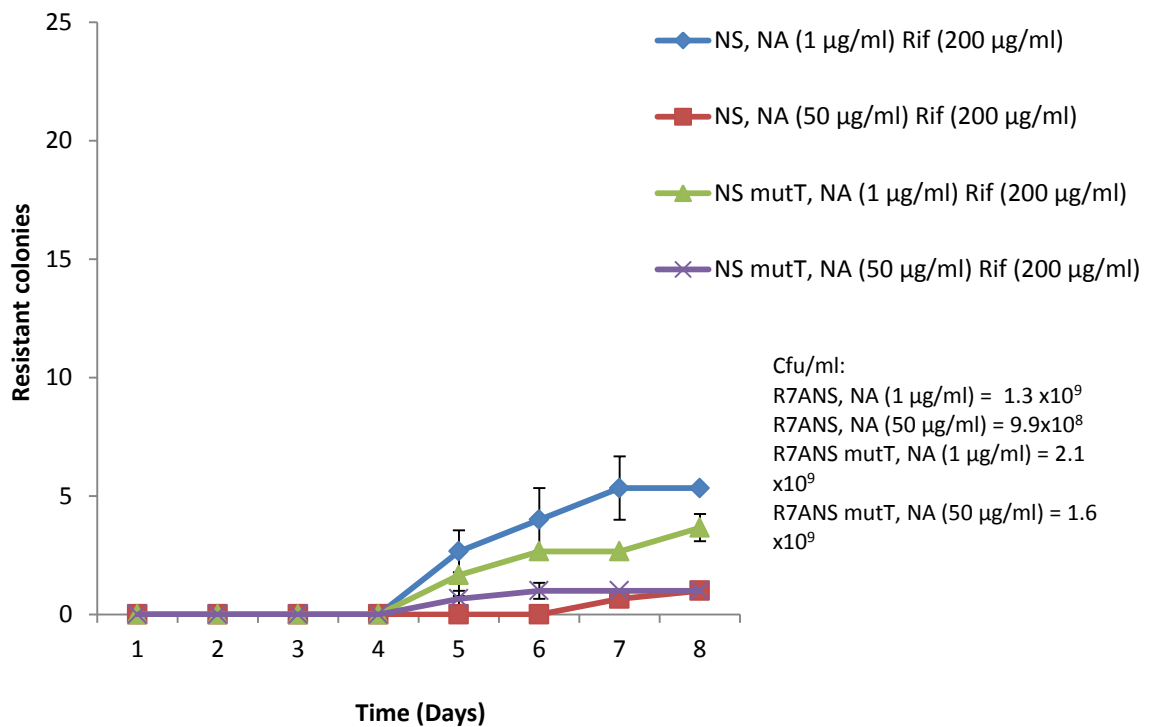


Figure 3.22 The cumulative appearance of rifampicin resistant colonies in *M. loti* R7ANS Δ *mutT* and *M. loti* R7ANS over time, performed as detailed for Fig 3.17.

3.6.4 Hydrogen peroxide sensitivity assay

The hydrogen peroxide sensitivity assay was performed on the various *mutT*-deficient *M. loti* R7A strains (R7A, R7ANS and R7A Δ dct backgrounds) in the same manner as described in section 3.5.4. The results from this assay showed no statistically significant difference between these 3 *mutT*-deficient strains in comparison to their equivalent wild-type strains (Figure 3.23). A p-value below 0.05 denotes that the difference between the two means is statistically significant, however, in this instance they all showed values above 0.05.

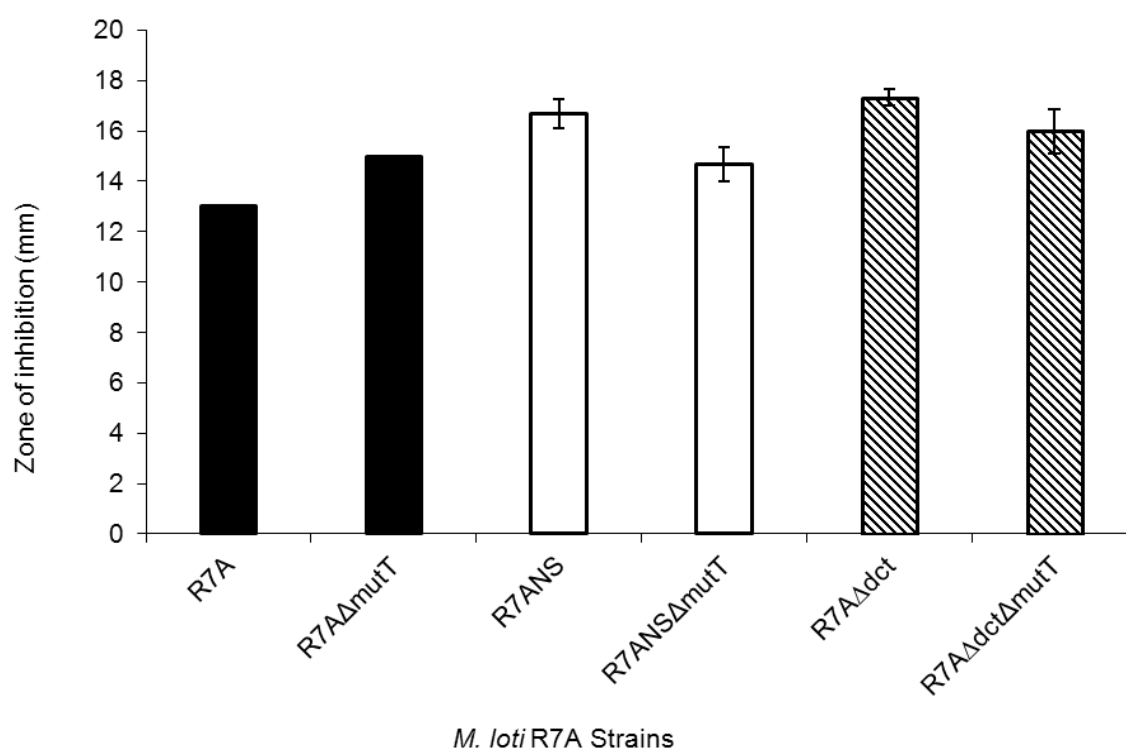


Figure 3.23 H₂O₂ zone of inhibition assay. Each bar represents the average diameter for triplicate measurements of the zone of inhibition formed around a disc containing 0.61 M H₂O₂ for each of the strains listed in the figure. Three *M. loti* backgrounds are represented: R7A (black), R7ANS (white), and R7A Δ dct (diagonal lines). Each strain is represented along with their *mutT* mutant. Asterisks indicate samples with significant deviation from the wild-type data of the same background strain (*P < 0.05).

3.7 MMR system mutants

3.7.1 Mutator phenotype of MMR system mutants of *R7AΔdct*

MMR is a system that exists to help maintain genome integrity. This system corrects mismatched base pairs that can occur through replication errors and chemical damage. MutS is a critical MMR system protein that has the role of recognition and binding of mismatched DNA.

The *M. loti* *R7AΔdct-mutS* mutant was tested by spread plating onto succinate media for the appearance of Dct⁺ colonies in the same way as detailed in section 3.3.1. The results showed a clear increase in the number of Dct⁺ revertants in the strain *M. loti* *R7AΔdct-mutS* compared with the control strain, *M. loti* *R7AΔdct* (Fig. 3.24).

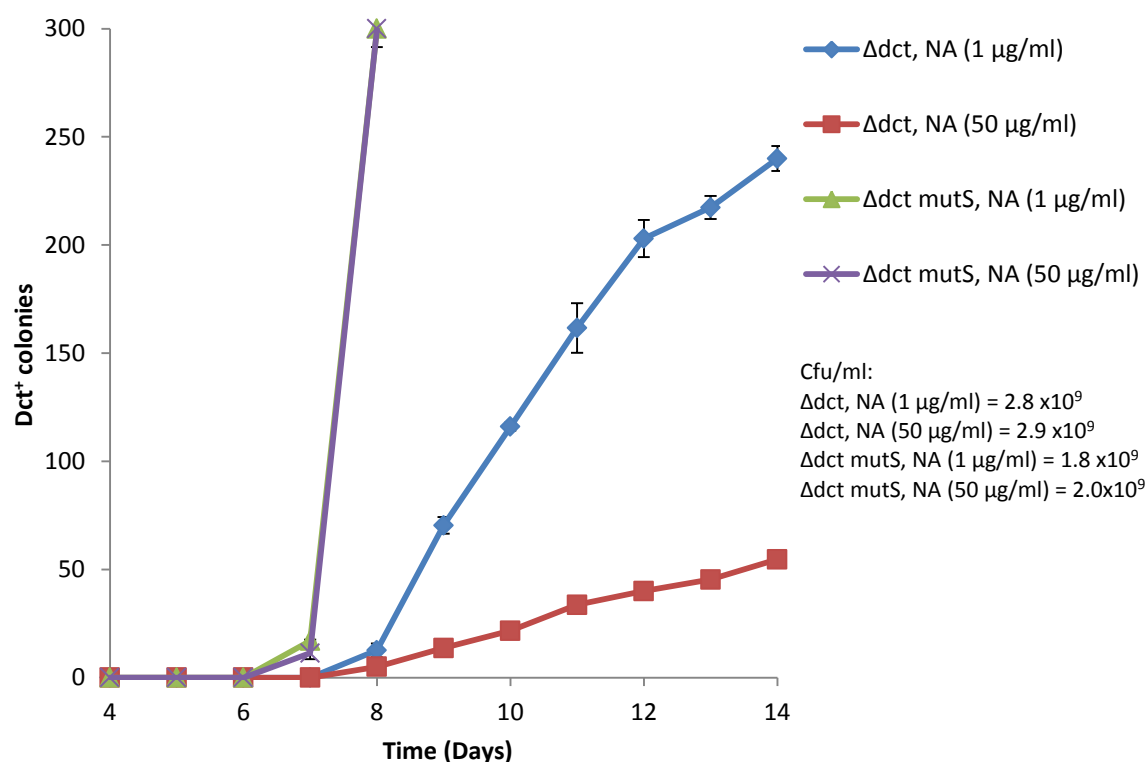


Figure 3.24 Cumulative appearance of Dct⁺ colonies in *M. loti* *R7AΔdct-mutS* and *M. loti* *R7AΔdct* over time at two concentrations of NA (1 μg/ml and 50 μg/ml). The *M. loti* *R7AΔdct-mutS* and *M. loti* *R7AΔdct* mutant shows a larger number of Dct⁺ colonies over time, indicating an increased mutation rate. Note that for *R7AΔdct-mutS* at both concentrations of NA, the number of colonies reached 300 at day seven making it difficult to distinguish the two separate lines.

3.7.2 Mutator phenotype of MMR system mutations in the *M. loti* R7ANS background

The *M. loti* R7ANS-*mutS* mutant was tested by observing the frequency of appearance of Dct⁺ colonies by spread plating onto succinate media, as described above in section 3.3.1. Overall, the *mutS* mutant strain showed an increase in the number of Dct⁺ colonies in the presence of NA at 1 µg/ml and at 50 µg/ml compared with the R7ANS control strain at both concentrations (Fig. 3.25). R7ANS showed several colonies arising on media containing an excess of NA (50 µg/ml) compared with no revertants on media containing 1 µg/ml. When plated on succinate medium containing an excess of NA, the *mutS* mutant gives rise to a large number of Dct⁺ revertant colonies.

In *mutS* mutant strains, an increase in mutation rate often reflects replication error, however, the evidence suggests that this is not the case. If the mutator effect was caused by replication error then the *mutS* mutant in the presence of 1 µg/ml of NA would also be expected to produce a large increase in mutation rate, similar to that observed for the *mutS* mutant in the presence of 50 µg/ml, which did not occur.

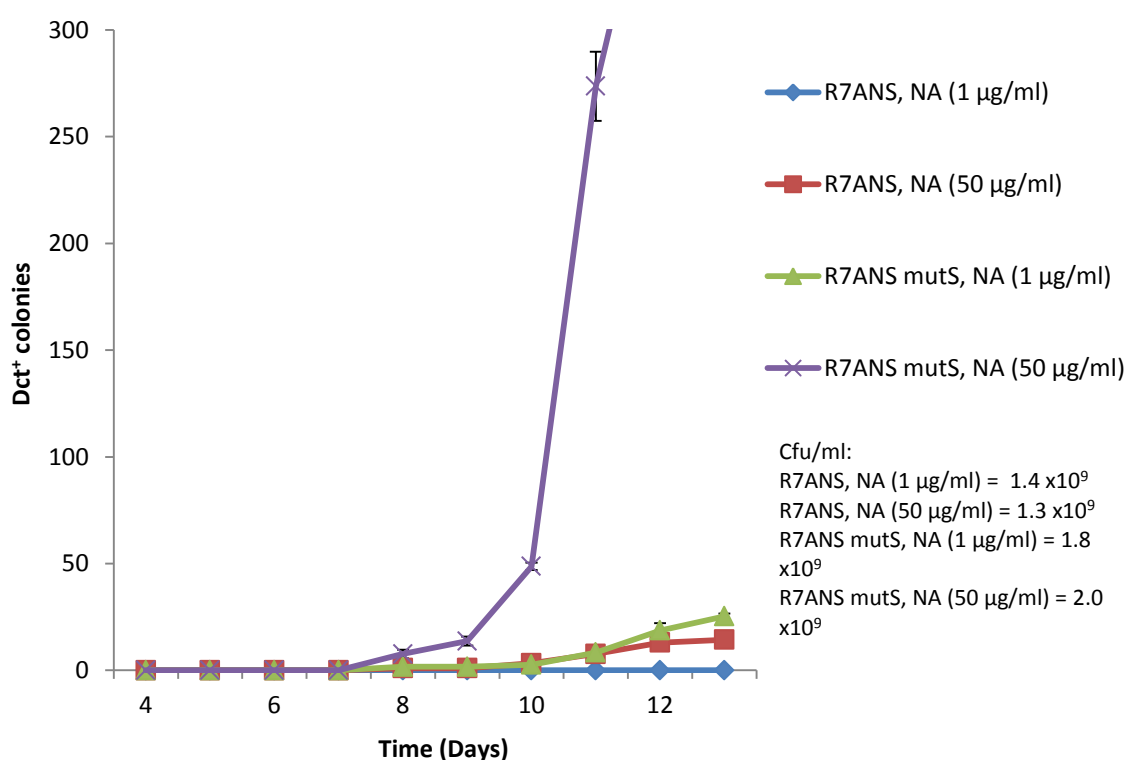


Figure 3.25 The cumulative appearance of Dct⁺ colonies in *M. loti* R7ANS-*mutS* and *M. loti* R7ANS over time for two different concentrations of NA (1 µg/ml and 50 µg/ml). The R7ANS-

mutS mutant strain showed a significant increase in the number of Dct⁺ colonies, showing a higher mutation rate in this strain. This effect was dramatically increased in the presence of an excess of NA, indicating a role in this mutator phenotype.

3.7.3 Appearance of antibiotic resistance colonies on rifampicin with the R7ANS *mutS* mutant strain

In this experiment strains *M. loti* R7ANS-*mutS* and *M. loti* R7ANS were plated onto G/RDM containing 200 µg/ml of rifampicin (Figure 3.26). A ~100-fold increase in the number of Rif resistant colonies was observed for the *mutS* mutant strains, likely due to mutations occurring pre-plating due to the lack of a functional MMR system in these strains. The mutations occurring pre-plating would explain why the majority of the colonies arose after four days. The number of resistant colonies observed between the different concentrations of NA was essentially the same.

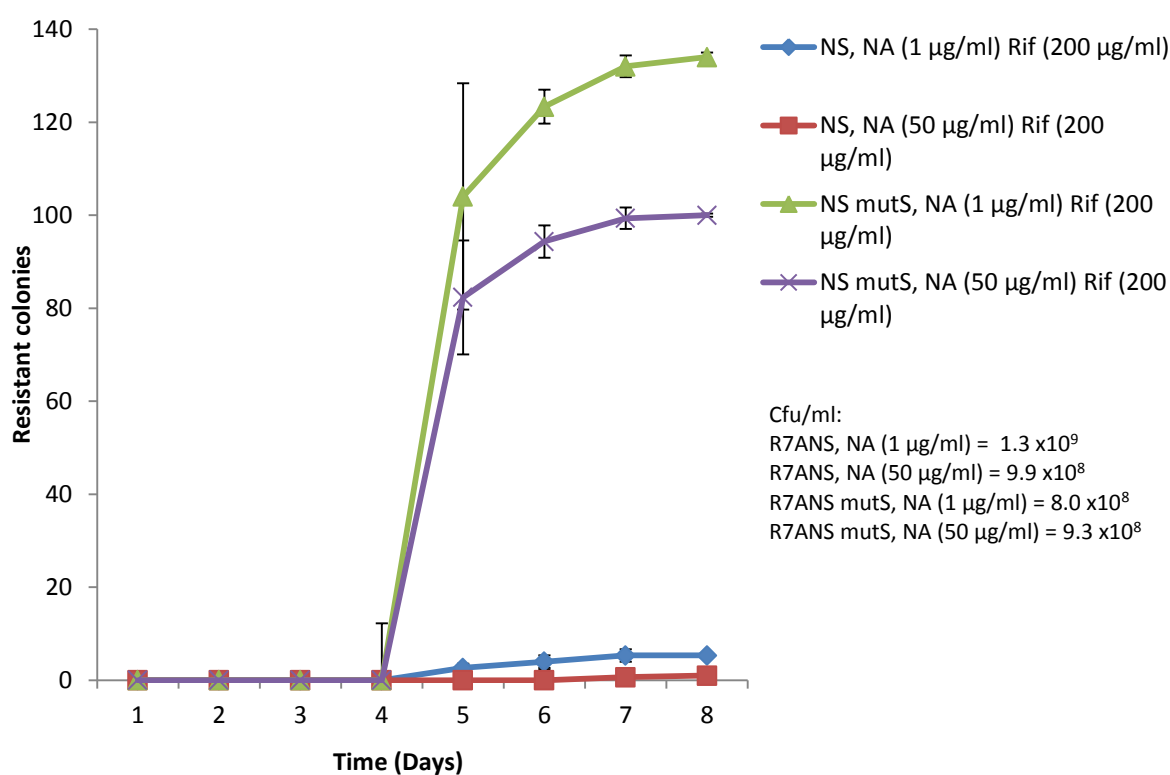


Figure 3.26 The cumulative appearance of rifampicin resistant colonies in *M. loti* R7ANS-*mutS* and *M. loti* R7ANS over time grown on G/RDM containing rifampicin (200 µg/ml), at two different concentrations of NA (1 µg/ml and 50 µg/ml). The cultures were standardised to an OD₆₀₀ of 1, and 100 µl was plated.

4

Discussion

4 Discussion

4.1 The role of the *dct* and *nad* genes in SIM

Previous experiments in our laboratory identified NAD-mediated SIM whilst studies were being carried out on non-symbiotic *Mesorhizobium* strains (Sullivan, Eardly *et al.* 1996). These non-symbionts should not be capable of succinate utilisation as they contain non-functional *dct* genes; however, when plated under these conditions it was found that gain-of-function

mutations arose in the *dctB* or *dctD* genes which allowed the *dctA* symporter to be expressed, meaning that the organism could then utilise succinate. The occurrence of these mutations was found to require the *nadABC* genes present on a plasmid in these strains. Although the mechanisms regulating NAD homeostasis in *Mesorhizobium* are unknown, it is plausible that NAD⁺/NADH homeostasis is lost under stressful conditions i.e. nutrient limitation. NADH is toxic (Grose, Joss *et al.* 2006) so a build-up of this could lead to oxidative stress and a hypermutator state until a mutation allowing growth occurs, at which point the mutation will become fixed and NAD homeostasis will be restored. The hypermutation state that would be achieved under stress would result in a genome-wide increase in non-selected mutations. Previous work investigated the role NAD plays in the development of SIM in *Mesorhizobium* and led to a proposed mechanism where NAD facilitates DNA damage by providing a reducing agent in the form of NADH (Cabiscol, Tamarit *et al.* 2010), which recycles Fe³⁺ back to Fe²⁺, increasing Fe²⁺ availability for the Fenton reaction (Ashbrook 2012). It is well known that cells that are non-growing or stressed exhibit elevated levels of ROS (Jakubowski, Biliński *et al.* 2000; Yu and Chung 2006; Sherman and Gagneux 2011), and under such conditions, as in the succinate reversion assays, it is likely that *M. loti* is experiencing elevated levels of ROS (Ashbrook 2012). Furthermore, NADH shows an increased affinity for reduction of Fe³⁺ which is approximately 16-fold that of NADPH (Brumaghim, Li *et al.* 2003) allowing for rapid recycling to Fe²⁺, providing iron for the Fenton reaction. A crucial aspect of this mechanism is the involvement of oxidative stress and ROS in the cells that are leading to the observed increase in mutagenesis. In order to substantiate the proposals from previous work (Ashbrook 2012; Sullivan Unpublished data), it was necessary to provide evidence that oxidative stress is responsible for the mutagenesis occurring in these cells undergoing SIM. Since there is little information on such mechanisms in the literature, this study aimed to expand on the previous evidence supporting this hypothesis. To test the theory that NAD-mediated SIM is due to increased oxidative stress and hypermutation, we have isolated mutants defective in DNA

repair and oxidative stress response genes. We hypothesised that such mutants should show an increased rate of NAD-mediated SIM.

4.2 Selection of genes to be mutated and construction of mutants

The selection of genes was carried out using Blast and Rhizobase as described in the results section. At the time, the *M. loti* R7A genome was yet to be sequenced so the selection of the seven genes chosen was carried out based on the *M. loti* MAFF303099 strain sequence. Although this is not a perfect scenario, *M. loti* R7A shows approximately 98% sequence identity with *M. loti* MAFF303099. The *M. loti* R7A genome is now sequenced and the presence of the chosen genes has since been confirmed.

IDM was the method chosen to construct single gene knockouts for 6 of the 7 genes. However, due to size of the putative *mutT* gene, this mutant was constructed as a markerless deletion mutant. IDM was chosen because it is a simple and effective method. However there are problems with this method. Disruption of the target gene involves insertion of the entire vector somewhere into the gene of interest, which completely removes its function. The problem of using IDM is that it creates polar mutations. Complementation analysis was performed by introducing a vector containing a wild-type copy of the deleted gene into the mutant. This should distinguish whether any observed effects are being caused by the target gene deletion, in which case wild-type function should return, or if it is actually a down-stream effect caused by the polar mutation.

Each gene was mutated in three different *M. loti* R7A strain backgrounds, wild-type R7A, R7ANS and R7A Δ dct. Each of these three strains has slightly different characteristics that made it important to use all three. The wild-type R7A was mainly used as a control strain. R7A contains the symbiosis island, which contains the *nadQABC* and *dct* genes. R7A Δ dct contains the symbiosis island but the *dct* genes present on this island have been deleted. R7ANS is missing the symbiosis island altogether. In succinate utilisation experiments R7A Δ dct was used to test the mutant phenotypes as it still contains the *nadQABC* genes which are required for the hypermutator state, but lacks the functional island copy of the *dct* genes and this is the cause of stress when the organism is plated on succinate. R7ANS was also used since the lack of island genes means that it does not contain functional *dct* genes or the *nadQABC* locus, and therefore should not undergo an increased mutation rate, unless excess levels of exogenous NA are present.

4.3 Catalase (*katG* and *katE*) mutants

The *katE* and *katG* genes were originally identified in *E. coli* and shown to encode a monofunctional catalase and a bi-functional catalase/peroxidase, respectively (von Ossowski, Mulvey *et al.* 1991). Under normal conditions with no stress, a complete lack of catalases appears to confer no decrease in growth rate or viability compared with a parent strain (Loewen 1984). However, it has been demonstrated that *E. coli* mutants deficient in these catalases dramatically increase their spontaneous mutation rate in response to oxidative stress in the form of H₂O₂ (Abril and Pueyo 1990), as well as lethal sensitivity to H₂O₂ by up to 50 – 60- fold (Loewen 1984; Abril and Pueyo 1990).

The *katE* and *katG* genes have been characterised for *M. loti* MAFF303099 (Hanyu, Fujimoto *et al.* 2009) and were shown, similarly to *E. coli*, to encode a monofunctional catalase and a bi-functional catalase/peroxidase, respectively. Hanyu *et al.* described a modest decrease in the growth rate of the *katG* mutant for both solid and liquid media when compared with the *katE* mutant and the wild-type strain. Various assays were performed under free-living and symbiotic conditions. They revealed minimal phenotypic variation of the *katE*-deficient strain from wild-type, especially in free-living conditions. The main effect of the *katE* mutant occurred *in planta*, showing a decrease in nitrogenase activity. When assessing survival rates of these mutants over time, mid-exponential cells were diluted and inoculated into TY medium and then grown to maximum cell density. The *katE* mutant began to lose viability soon after reaching maximum density, whereas wild-type MAFF303099 maintained viability for at least 24 hours longer, leading to the conclusion that *katE* appears to play a role in survival during stationary-phase. The authors also performed H₂O₂ sensitivity assays. Although no significant effect occurred in the *katE*-deficient strain, a significant increase in sensitivity of the *katG*-deficient strain when compared with *M. loti* MAFF303099 was observed. Furthermore, complementation decreased the sensitivity slightly below what was seen in the wild-type strain, which was explained by the multiple copy number of the plasmid carrying the complemented gene. Hanyu *et al.* conclude that *katG* played the main role in detoxification of internal H₂O₂ as well as exogenously added H₂O₂, whereas *katE* has a role of optimising survival in stationary-phase along with the capacity for nitrogen fixation.

The functional difference of the two catalases is likely explained by the regulation of gene expression which varies between the two. In *M. loti* MAFF303099, *katG* is encoded next to and divergently transcribed from a gene encoding a putative OxyR-like transcriptional regulator. When gene expression was assessed, *katG* expression increased approximately two-fold under

aerobic conditions, in contrast with *katE* expression which remained unchanged. In *E. coli* it is known that *katG* is under the control of OxyR, whereas *katE* is regulated by the stationary-phase sigma factor RpoS (Storz, Tartaglia *et al.* 1990; Lange and Hengge-Aronis 2006). Given the sequence similarities of KatE and KatG between *E. coli* and *M. loti* MAFF303099 (50% for KatE and 61% for KatG), it seems reasonable to suggest that the enzymes play very similar roles in each species.

The *katE* and *katG* mutants constructed were subjected to a series of phenotypic assays to determine their function in *M. loti*. The sequencing of these genes in *M. loti* R7A revealed 99% sequence identity at the nucleotide level with those in *M. loti* MAFF303099, indicating they will have the same function. For the H₂O₂ sensitivity assay, *M. loti* MAFF303099 was used as a control to try and replicate the published study, in order to validate the findings. The H₂O₂ assay performed in this study was based on the same experiment detailed by Hanyu *et al.* and the results reflect the results published in their paper, revealing a large increase in sensitivity of the *katG*-deficient strain when compared to wild-type. Similarly in this study, complementation of *katG* mutants in *M. loti* R7A, R7ANS and R7A Δ dct also decreased the sensitivity to hydrogen peroxide to a level below that of the wild-type strains, likely caused by the multiple copy numbers of the vector used for complementation.

Following this experiment mutation rates were assessed in both the *katE* and *katG* under conditions of starvation, using the succinate utilisation assay. Firstly the catalase mutants were assessed on succinate in the R7A Δ dct background. The conditions of starvation gave rise to a marked increase in the rate of appearance of Dct⁺ mutants over time in the *katG* mutant (Figure 3.15A). By day 11 on succinate medium containing 1 μ g/ml NA, the *katG* mutant when plated at the 10⁻³ dilution ($\sim 10^8$ cells) had given rise to about 300 colonies. Although the high level of 50 μ g/ml NA slightly represses the mutational effect in wild-type cells, it only took 12 days on this medium to reach 300 colonies. In comparison, the parental strain took 14 days to reach 150 and 70 colonies at low and high levels of NA, respectively. In contrast, *katE* appeared to show a slight decrease in number of mutants compared with the wild-type, due to a delayed onset of mutant appearance (Figure 3.15B). As discussed earlier with reference to the literature, it is not surprising to see a lack of effect under these circumstances; *katG* is proposed to be solely responsible for detoxification in growing cells in response to internal or external H₂O₂.

The succinate mutation assay was also performed using the strain R7ANS-*katG* (Figure 3.16). Removal of the mobile symbiosis island, and therefore NAD genes, should remove the

proposed NAD-induced oxidative stress occurring during starvation on succinate medium. However, as detailed earlier, a high level of NA can reproduce the effect that is conferred by the presence of the *nad* genes. The results obtained reflected this. At 1 µg/ml NA, not a single mutant appeared for the parent strain over the 13 day period, whereas for the *katG* mutant only 20 colonies appeared. Importantly, these mutants first appeared at day 8 and did not increase in number thereafter. This suggests they may have been pre-existing in the culture prior to plating, which may be explained by production of internal H₂O₂ under normal growth conditions. However, at a level of 50 µg/ml NA which has been shown mimic the effect caused by the *nad* genes (Ashbrook 2012), the parent strain gave rise to 15 mutants while the *katG* mutant reached 130 mutants. Succinate mutant appearance in the *katG* mutant was biphasic, with the first phase from days 8 to 10 similar to that seen under 1 µg/ml NA, while the second phase of exponential increase started at day 10 and likely reflects increased oxidative stress under starvation. This result shows clearly the effect that NAD has on a cell under starvation, and the response of the mutant, which is highly sensitive to oxidative stress, demonstrates that oxidative stress induces the observed mutator phenotype.

The lack of effect of the *katE* mutation on succinate mutation in the three *M. loti* R7A-derived backgrounds is consistent with its lack of change in H₂O₂ sensitivity and with the observations of Hanyu *et al.* In their paper they reported that the main effect of the *katE* mutant occurred during symbiosis, which was not assessed in this study. The results with the *katE* mutant support the findings that KatG and not KatE is important for the detoxification of ROS in *M. loti* cells under stress.

To determine if the mutants underwent an increase in spontaneous mutation rate in growing cultures, the number of rifampicin-resistant mutants in glucose-grown cultures was determined. The strain *M. loti* R7ANS-*katG* showed a smallish but significant increase in number of Rif-resistant colonies compared to the parental strain (Figure 3.17). As detailed previously, KatG is responsible for protection against oxidative stress in the form of H₂O₂. Since H₂O₂ is also produced during normal aerobic respiration due to leakage of single electrons from the respiratory chain, KatG plays a role during normal growth conditions. For example, González-Flecha *et al.* (1995) showed that mutational suppression of KatG, but not KatE resulted in an increase in the intracellular H₂O₂ concentration (González-Flecha and Demple 1995). Therefore, although the cells placed on media containing Rif have not been subjected to conditions of starvation and stress, it was not surprising to see a slightly elevated mutation

rate in the *katG* mutant strain, reflected in both Rif^R and Dct⁺ mutants arising during growth, as metabolic processes during aerobic growth provide a source of H₂O₂.

4.4 GO mutants

The GO system has been investigated thoroughly in a number of different organisms and appears to be highly conserved across many species. In *E. coli* it is made up of two DNA glycosylases (MutM, and MutY) as well as a pyrophosphohydrolase (MutT). Mutant strains missing the GO system genes have been shown in the literature to confer significantly increased mutation rates under conditions of stress. These three proteins have been detailed previously in section 1.8.1

Since the GO system is so important for survival against oxidative stress in *E. coli*, it seemed like an appropriate system to target for mutagenesis in this study to provide further evidence of the role of oxidative stress in the mutator phenotype. Sequence similarity was analysed between *E. coli* and *M. loti* MAFF303099 which showed that all three of these genes were very likely to share the same function in *M. loti*.

All three GO system mutants in the R7A Δ dct background were plated on succinate medium. Both MutT and MutY behaved as expected, showing a significantly increased reversion rate to Dct⁺ compared to the parental strain (Figure 3.20A and 3.20B). Both of these strains reached 300 colonies within 8 – 9 days after plating, yet the parental strain never made it higher than 250 over the full two-week period. In contrast with those results, MutM appeared to only deviate very slightly from parental strain in the presence of 1 μ g/ml NA, and showed no difference with 50 μ g/ml NA (Figure 3.20C). Although the literature shows that both MutM and MutY confer similar mutations and rates of mutation in *E. coli*, it appears that MutM may have a less important role in *M. loti* R7A. Alternatively the putative *mutM* gene in *M. loti* may be mis-annotated or there may be a duplicate of the gene present. However the latter possibilities seem unlikely as only one homologue of *E. coli* MutM was revealed by BlastP searches of the *M. loti* genomes and using that sequence (MII5585) as a query did not reveal further homologues.

Further work was done with *mutT* as a representative of a GO system mutant. A *mutT*, *mutM*, *mutY* triple mutant would have been ideal, but there was not time to construct and confirm it for the purpose of this study. In the R7ANS background (Figure 3.21) there were no revertants at 1 μ g/ml NA and only a few in the presence of 50 μ g/ml. In the R7ANS *mutT* strain at 1 μ g/ml NA approximately 10 revertants arose over the course of the experiment, but as expected the mutation frequency significantly increased for the mutant strain in the presence of 50 μ g/ml NA, supporting the hypothesis of increased oxidative stress in starved cells exposed to excess NA.

Unlike the *katG* mutants, when the *mutT* mutant strains were plated in the presence of H_2O_2 they did not show any difference in sensitivity to H_2O_2 (Figure 3.23). At first glance this result does not seem to fit the idea that these mutants are undergoing increased oxidative stress. However, the *mutT* mutant strains still have a functional *katG* gene, and therefore are still producing catalase that breaks down H_2O_2 . The role of *mutT* is to hydrolyse 8-oxo-dGTP to 8-oxo-dGMP, reducing the levels of oxidised guanine (Fowler and Schaaper 2006). The assay does not involve cells under carbon starvation and so the presence of a functional KatG is likely sufficient to decompose any H_2O_2 before it can result in the formation of 8-oxo-dGTP. It would be interesting to investigate the effects of a *katG/mutT* double mutant to determine if that mutant is more sensitive than the *katG*-deficient strains alone.

In the Rif assays G/RDM was used, removing conditions of starvation. The lack of starvation means that there should be no oxidative stress induced, which combined with the bactericidal activity of Rif led to the hypothesis that there should be no increase in resistant colonies occurring post-plating in the mutant strains when compared with the wild-type parental strain. Strain *M. loti* R7ANS Δ *mutT* revealed at most a small increase in the number of Rif resistant colonies (Figure 3.22), when compared with wild-type R7ANS (Figure 3.21). This is an expected result, as *M. loti* R7ANS Δ *mutT* should only show increased mutation rates under conditions where oxidative stress is being induced and any mutants must appear pre-plating as rifampicin is a lethal selection. What this result shows is that these strains are not just constitutive mutators, as shown by the lack of Rif resistant colonies. They need to be placed under conditions of stress for mutation to occur, as in the succinate assay where starvation is driving NADH/NADPH imbalance which leads to oxidative stress.

4.5 MMR mutants

The MMR system is a highly conserved system that exists to maintain genome integrity. Although the main source of mismatched base-pairs is replication error, it is known that biological processes can account for a significant amount (Fukui 2010). MutS plays a crucial role in MMR, as it recognises and binds mismatched DNA.

In *E. coli* it has been shown that for the Cairns system, constant nicking at *oriT* on the F'128 episome gives rise to ssDNA upon which replication forks become stalled. This is followed by RecA invasion and subsequently DSB as well as SOS induction, leading to error-containing newly synthesised DNA due to error prone polymerases (Stumpf, Poteete *et al.* 2007). The MMR system then corrects these errors in the daughter strand. The number of errors that arise are believed to overwhelm the MMR system and give rise to SIM. Following the Cairns system, SIM was investigated in many other organisms. Interestingly in *B. subtilis*, SOS induction of *recA* was found to be completely unnecessary in the induction of mutagenesis. Furthermore, MMR was shown to be very important in SIM in *B. subtilis*, and strains deficient in MMR showed an increase in stationary-phase mutations (Pedraza-Reyes and Yasbin 2004). Similarly, our laboratory has found that mutational inactivation of *recA* in *M. loti* R7A has no effect on succinate mutation rate (Ashbrook 2012). As is seen with *B. subtilis*, it appears that in *M. loti*, DSB is not the significant cause of DNA damage and therefore the DSB mechanism proposed in the Cairns system cannot be the mechanism leading to SIM in this organism.

As with the other mutants for the succinate utilisation experiment, a *mutS* mutant in the Δdcl background gave rise to a large number of revertant colonies (Figure 3.24). When this experiment was performed in the R7ANS strain (Figure 3.25), the results in the presence of 50 $\mu\text{g/ml}$ NA were even more dramatic than those for *katG* and *mutT* mutants. Although the MMR system is not explicitly responsible for oxidative damage reduction, it is involved in the repair of oxidative stress induced DNA damage which has been clearly demonstrated in *M. loti*.

Following the succinate utilisation assays, R7ANS-*mutS* showed a significantly different result to that of *M. loti* R7ANS Δ *mutT* and *M. loti* R7ANS-*katG* in the presence of Rif. This experiment was carried out exactly the same way as for strains *M. loti* R7ANS-*katG* and *M. loti* R7ANS Δ *mutT*, however this time, a large increase in the number of Rif resistant colonies was observed between *M. loti* R7ANS-*mutS* and wild-type *M. loti* R7ANS (Figure 3.26). As with the previous Rif assays, R7ANS only produced a few colonies, up to about five over the eight day incubation. R7ANS-*mutS* produced approximately 100-130 colonies over the same period, with the majority appearing after only four days, which is consistent with wild-type growth-rate,

indicating that these mutations had occurred pre-plating. The incubations only lasted eight days as colony formation had already reached a plateau.

Since MutT and KatG both provide roles in defence explicitly to oxidative stress, it is expected that under conditions where there should be no oxidative stress, no increase in mutation should be observed. MutS plays a significantly different role; MutS is part of the MMR system which is detailed in the Introduction. This system has various defensive roles, many of which are unrelated to oxidative stress. Strains defective in aspects of MMR are shown in the literature to undergo increased spontaneous mutation rates (Miller 1996). A paper investigating *mutS* reported a 100-fold drop in spontaneous mutation frequency in all cases when strains containing a mutant *mutS* were complemented with the wild-type allele (Siegel and Bryson 1967). Therefore, given the nature of *mutS* mutation, the large number of spontaneous Rif resistant mutants that occurred pre-plating is expected, even in the absence of oxidative stress.

What is important to note, is that even with the large number of Rif resistant colonies that arose for the *mutS*-deficient strain, the number of succinate mutants that occurred during conditions of starvation, i.e. on succinate, was much higher. It is important to note that the Rif resistant assays were plated without dilution, however, the succinate reversion assays were plated at the 10^{-3} dilution. It may look as if there is little difference between *M. loti* R7ANS-*mutS* on succinate compared with *M. loti* R7ANS-*mutS* in the presence of Rif (Figure 3.25 and 3.26 respectively), however there are approximately 1000-fold less cells plated for the succinate reversion assay (Figure 3.26) which illustrates the severity of the effect that oxidative stress is having. Comparing the R7ANS strains on media containing succinate against media containing Rif not only shows the intensity of the increase in mutation rate, but also addresses another key issue. Since strains deficient in the genes chosen for mutation in this study are known mutators, it is important to see that the spontaneous mutation rate is much lower (at least 1000-fold) when conditions of starvation, and therefore oxidative stress, are absent. When looking between these results it becomes impossible to argue that the high mutation frequency is simply due to these strains being constitutive mutators. It is clear that the starvation conditions are driving this SIM.

4.6 Comparison to SIM in other bacteria

The mechanism of SIM that is hypothesised in this study differs from that in the Cairns system, however it shows many similarities for SIM described in several other systems where oxidative stress has been implicated. In *B. subtilis*, extensive research into the mechanism of SIM was carried out. Transcription-associated mutagenesis was revealed as the mechanism occurring in *B. subtilis* (Davis 1989; Pybus, Pedraza-Reyes *et al.* 2010). A strain deficient in Mfd proved to decrease stationary-phase mutagenesis, with a likely cause of *mfd*⁺ mediated mutagenesis being oxidative stress (Ross 2006). Furthermore it is theorised that *B. subtilis* differentiates a subpopulation of hypermutable cells, which achieve an increase in mutation through suppression of DNA repair systems (Sung and Yasbin 2002). *B. subtilis* contains a complete MMR and GO system and knockout mutants were constructed to investigate the effects on stationary-phase mutagenesis. A strain lacking the *mutSL* operon significantly increased the number of stationary-phase mutants occurring (Pedraza-Reyes and Yasbin 2004). When complementation failed to completely remove the mutator phenotype, investigation was carried out on the effects of the GO system. Similar to the results found in our study, Vidales *et al.* found a dramatic increase in mutation rates for the various GO system mutants that were constructed. The *mutSL* operon was then overexpressed in a GO system triple mutant with a similar effect to that of complementation. The various results revealed in *B. subtilis* reflect similar outcomes to those observed in this study, and provide compelling evidence that oxidative stress plays a crucial role in SIM in this species.

Another example of SIM was investigated in *P. putida*, which also provided similarities with the results found in this study. The experimental system used in *P. putida* assessed mutation rates under conditions of starvation by using the *pheBA* genes as a reporter, the presence of which conferred the ability to use phenol as a carbon source (Saumaa, Tover *et al.* 2002). The authors found that over time, the mutational pathways may vary, depending on the time spent under conditions of starvation. ROS was found to play a direct role in SIM for this bacterium; knockout mutants deficient in *mutM*, *mutY*, and *mutT* were constructed and examined using two assays. The mutation frequencies of these mutants increased dramatically, with the exception of *mutM*, which is consistent with the results obtained in the current study. Further investigation sought to discover the effect RpoS would have on the mutator phenotypes observed. In the *rpoS*-deficient strain, the rate of Rif^R mutation was significantly increased and, coupled with decreased survival, damage through ROS appeared to be the underlying cause (Kivisaar 2010). More evidence was gained from the *rpoS*-deficient strain by analysis of SOD and a catalase, KatB, which is positively regulated by RpoS. SOD levels were approximately 5-fold

lower in the *rpoS*-deficient strain, overexpression of SOD as well as the complementation using KatA, the housekeeping major catalase in *P. putida* (Hishinuma, Yuki *et al.* 2006), both improved survival and reduced base substitution mutation frequency (Tarassova, Tegova *et al.* 2009). As with both *B. subtilis* and *M. luti* R7A, carbon-starved stationary-phase *rpoS*-deficient *P. putida* underwent increased mutation rates due to oxidative DNA damage. The lack of RpoS also decreased repair fidelity and DNA replication due to oxidative damage of enzymes and translational errors of proteins (Tarassova, Tegova *et al.* 2009; Kivisaar 2010). Both of these systems, as well as that described in the current study, have contributed considerable evidence that ROS is the main cause of damage where SIM is occurring.

4.7 Conclusions

It is widely accepted that cells in stationary-phase or under stress possess elevated levels of ROS, however two key questions need to be settled. Firstly, is oxidative damage from ROS the cause of the mutagenesis in this system? Secondly, is NAD modulating the level of oxidative damage?

The systems that have been altered in this study specifically targeted oxidative stress repair systems. The three systems chosen for manipulation were the GO system, MMR system and a catalase, which have been explained in detail previously. Mutant strains constructed that are missing aspects in each of the three systems showed significantly increased mutation rates compared to non-mutant parental strains when placed under conditions of starvation. Mutants defective in the catalase KatG and two of the three GO system genes that were inactivated underwent large increases in succinate revertant colony appearance under conditions that were hypothesised to induce oxidative stress, providing compelling evidence that oxidative stress was rife throughout the cells plated onto succinate containing media. Mutants defective in the MMR system gene tested, *mutS*, also showed similar increases in mutation. Although this system is not specifically related to oxidative stress, it can play a crucial role under such conditions (Fukui 2010). Strains that lack MutS are known for high rates of spontaneous mutation (Siegel and Bryson 1967), however, as discussed earlier, when assessing the mutation rate on media containing Rif the number of mutants was at least 1000-fold less than the number that occurred under conditions of starvation. Therefore although spontaneous mutations do arise in this strain, it is likely to be a statistically negligible proportion of those that arise due to conditions of oxidative stress. The severely elevated mutation rates that occur in strains that are deficient in some aspect of their oxidative stress

response systems is a clear indicator that the cause of mutation under these conditions is ROS generated through oxidative stress in the cell.

A possible argument that could be made to the comparison of mutation rates with rif and on succinate is that there could be other factors affecting the outcome, such as target size. Instead, a key comparison is looking at the R7ANS mutant on 1 µg/ml NA compared with 50 µg/ml; a mutant in the R7ANS background from each of the three systems produced a small number of revertants in the presence of minimal NA, however, none of the three strains produced more than 20. This was expected; even without the mutator effect that is conferred by either the *nadQABC* genes or 50 µg/ml NA, these mutants exhibit elevated mutation rates. However, in the presence of excess NA, the mutation rate is significantly increased; the *katG*-deficient strain, *mutT*-deficient strain, and *mutS*-deficient strain produced approximately 130, 70, and 300 Dct⁺ revertant colonies over the 14 day incubation on succinate, respectively.

NAD modulation of this effect has been observed throughout these experiments with overwhelming evidence that NAD is collaborating with ROS to induce this SIM. Mutant strains in the R7ANS background (deficient in genes for NAD *de novo* biosynthesis) experience minimal mutation rates unless supplemented with an excess of NA providing a substrate for the production of NAD through the salvage pathway. The equivalent assays carried out in the R7AΔ*dct* background results in a significant mutation rate without additional NA, as this strain contains the NAD biosynthesis genes. This study has provided evidence that SIM in *M. loti* requires two concurrent factors: The cell must possess the ability to produce NAD, and this must coincide with a state of oxidative stress inside the cell.

The predicted mechanism this study is supporting is that under stress, there are elevated levels of both ROS and NADH, leading to HO[•] formation via the Fenton reaction and this damage leads to wide-spread mutation. Deleterious mutations are repaired and beneficial mutations are fixed, at which point the stress is removed through acquisition of this beneficial mutation and homeostasis is restored.

Although the results from this study seem clear, further experimentation could be carried out to build upon the evidence gathered thus far. Only one gene was targeted for each mutant constructed in this study. It would be useful to investigate the effects of a double or triple mutant, e.g. a complete GO system knockout mutant, and then observe the phenotype of that strain under the conditions examined throughout this study. Often in the literature there were

instances where a double or triple mutant would show a mutation rate higher than the sum of the mutation rates of the single mutants.

Since the Fenton reaction is central to the generation of $\text{HO}^\cdot$ it would be invaluable to investigate the effects of an iron chelator and $\text{HO}^\cdot$ scavengers on the mutation rates of the various oxidative stress response mutants during stationary-phase. The use of these with these mutants should also decrease mutation rates observed under conditions of SIM, and the use of $\text{HO}^\cdot$ scavengers has previously been shown to reduce mutation rates for organisms exposed to various agents including H_2O_2 .

4.8 The wider implications of oxidative stress as a mechanism for adaptive mutation and SIM

With the use and misuse of antibiotics in medicine, there is a constant and increasing problem of drug-resistance among pathogens of many different species. Bacteria have a very short life-cycle which allows for rapid evolution and natural selection making the fight against antibiotic resistance difficult, to say the least. An exacerbation in this fight is the lack of new antibiotics discovered and the emergence and increasing spread of antibiotic-resistant bacteria (Walsh 2000). Antibiotic resistance can be achieved through a variety of different mechanisms, a few examples include: horizontal gene transfer (Davies 1997), selection of naturally occurring resistant variants, and increased mutagenesis/hypermotators (Andersson 2003; Chopra, O'Neill *et al.* 2003). Part of the problem is the lack of understanding of the specific actions of antibiotics that lead to cell death.

The specific killing mechanism of many antibiotics remains elusive. Based on their drug-target interactions, there are three major classes of bactericidal antibiotics: inhibitors of cell-wall biosynthesis; inhibitors of DNA replication and repair; and inhibitors of protein synthesis (Walsh 2000). A prominent theory that came to light recently proposed generalised induction of ROS as the lethal cause (Kohanski, Dwyer *et al.* 2007). The idea was that regardless of the specific mode of action, antibiotics were leading to Fenton-mediated $\text{OH}^\cdot$ formation. Furthermore it was noted that there was a strong correlation between ROS formation and fold-change in mutation rates after treatment using bactericidal antibiotics. It led the authors to suggest that ROS induced by these antibiotics is contributing to drug-induced mutagenesis (Kohanski *et al.*, 2010). In support of this, the addition of thiourea, a potent $\text{HO}^\cdot$ scavenger (Novogrodsky, Ravid *et al.* 1982), significantly reduced the mutation rate near untreated levels following exposure to norfloxacin, ampicillin, or H_2O_2 . These studies provided convincing evidence that ROS may be the cause of lethality in antibiotic cell death, however further research has investigated these studies and since, have provided compelling evidence to the contrary (Keren, Wu *et al.* 2013; Liu and Imlay 2013) indicating that ROS are, in fact, not responsible for antibiotic associated cell death.

Following the research implicating oxidative stress as one of the mechanisms by which bactericidal antibiotics kill bacteria, further research was carried out investigating multidrug resistance via radical-induced mutagenesis. Bactericidal antibiotics, including β -lactams, quinolones, and aminoglycosides are capable of stimulating production of ROS, which as

explained in the introduction, can cause various kinds of cell damage (Foti, Devadoss *et al.* 2012).

Although it now seems unlikely that antibiotics themselves kill via ROS, antibiotics at sublethal levels place cells under conditions of stress which is likely to induce mechanisms much the same as the one proposed in this study. It seems plausible that the mechanism described in this study, or one equivalent, could be one of the driving forces behind the development of antibiotic resistance.

A study published early in 2012 (Foti, Devadoss *et al.* 2012) was investigating why DinB overproduction was cytotoxic in *E. coli*. Inadvertently their work led to the insight that cell death caused by overproduction of DinB and by bactericidal antibiotics was the result of oxidation of guanine leading to the formation of 8-oxo-dGTP. They showed that under conditions of elevated levels of OH[•], it is not simply generalised oxidative stress leading to antibiotic-mediated cell death, but specifically 8-oxo-dGTP incorporation into the genome by Pol III, Pol IV, and Pol V, which leads to MutM- and MutY-dependent DSBs. Interestingly, when MutT was overexpressed, sensitivity to the three drugs tested was decreased. Although our study was not investigating the effect of bactericidal antibiotics, the results from this study are consistent with our work. The GO system appears to play a central role in defence, but under sufficient levels of bactericidal antibiotics it could lead to lethal MutM- and MutY-mediated DSB formation.

Developing our understanding of the way in which bacteria can mutate and adapt is crucial. The mechanism proposed in this study results in SIM via starvation stress, but as mentioned above, other triggers such as sublethal antibiotic treatment can lead to the same outcome. NAD metabolism and oxidative stress is a central part of the mechanism leading to mutagenesis in both our study and those investigating cell death by bactericidal antibiotics (Kohanski, Dwyer *et al.* 2007; Kohanski, Dwyer *et al.* 2010; Foti, Devadoss *et al.* 2012). This study has helped to elucidate a mechanism of SIM that involves NAD mediated oxidative stress in *M. loti*, providing insight as to how bacteria can increase their mutation frequency in response to environmental stress. It is critical for us to continue to expand our understanding of bacterial adaptation at the molecular level in order for us to develop and enhance antimicrobial therapies. NAD is a coenzyme found at all levels of life, and therefore this mechanism of SIM is likely to be conserved across other species. NAD metabolism is likely to provide an effective target in pathogens in order to reduce the occurrence of antibiotic

resistance, and NAD biosynthesis is already being investigated as a drug-target (Magni, Di Stefano *et al.* 2009).

5

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5 References

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6

Appendices

6 Appendices

6.1 Growth Media

All media and stock solutions were autoclaved (121°C at 15 psi) unless indicated otherwise.

6.1.1 LB: per litre

10 g tryptone
5 g yeast extract
5 g NaCl
15 g agar

6.1.2 TY: per litre

5 g tryptone
3 g yeast extract
2 ml of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (0.65 g/ml)

6.1.3 Rhizobium Defined Media (RDM): per litre of distilled water

G/RDM:

10 ml Salts solution
10 ml BTB
6 ml NH_4Cl
1 ml Trace elements
100 mg L-histidine

Once the histidine is dissolved, the pH is adjusted 6.5 – 7 with 2 M KOH. Following autoclaving the media is cooled at 55°C in a waterbath. Once cooled, 20 ml/L of 20% glucose, 10 ml/L phosphate solution, one ml/L vitamin solution, and any required antibiotics or antifungal agents are added.

Succinate RDM:

10 ml Salts solution
10 ml BTB
6 ml NH_4Cl
1 ml Trace elements
100 mg L-histidine

RDM stock solutions:

- **Carbon Source**

20% Glucose (1M): 40 g glucose/200 ml.

34% Sucrose (1M): 68.46 g sucrose/200 ml.

- **Phosphate solution (pH 6.5 - 7): per litre**

100 g K_2HPO_4

100 g KH_2PO_4

- **Salts solution: per litre**

25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

2 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

1.5 g FeEDTA

20 g NaCl

- **Bromothymol Blue (BTB): 2 mg/ml**

0.4 g BTB make up to 200 ml

- **Trace Elements:** per 200 ml
 - 3 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
 - 40 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
 - 50 mg H_3BO_3
 - 40 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$
 - 4 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 - 1 ml $\text{COCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2 g/L)
- **Vitamins:** (make up to 50 ml and filter sterilise)
 - 50 mg thiamine HCl
 - 100 mg Ca panthothenate
 - 1 ml biotin (1 mg/ml)
 - 18 g/L NH_4Cl

6.2 Buffers and Solutions

- **50 x Tris-acetate buffer (TAE):** per litre

Add the following to 600 ml of distilled water and then adjust to a final volume of one litre.

 - 242 g Tris base
 - 100 ml of 0.5 M EDTA
 - 57.1 ml of glacial acetic acid
- **Agarose gel:** (100 ml)

Dissolve 1 g of agarose powder in 100 ml of 1xTAE buffer.
- **Tracking dye:** (1 ml)
 - 300 μl glycerol
 - 300 μl bromophenol blue (10 mg/ml)
 - 400 μl Milli-Q H_2O

- **5 M NaCl:**

Dissolve 292.2 g NaCl in 800 ml H₂O. Adjust final volume to one litre.

- **1 M Tris (pH 8):**

Add 121.1 g Tris (hydroxymethyl) aminomethane to 800 ml distilled water. Add HCl to adjust to pH 8 (approximately 42 ml) and adjust to a final volume of one litre with distilled water.

- **20 x SSC (pH 7):**

Add the following to 800 ml of distilled water:

88.2 g tri-sodium citrate

175.3 g NaCl

Adjust to pH 7.0 with 10 M HCl, adjust volume to one litre using distilled water.

- **10% [w/v] SDS:**

Add 100 g sodium dodecyl sulphate (SDS) to 900 ml of distilled water and heat to 68°C to dissolve. Once cooled, adjust the pH to 7.2 with a few drops of concentrated HCl. Adjust to a final volume of one litre with distilled water.