Genetic basis of the activation of the cryptic *dct* genes in *Mesorhizobium loti*

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Abstract

Previous studies showed that, although non-symbiotic mesorhizobia carry a copy of the *dctABD* genes responsible for C_4-dicarboxylate transport, most strains isolated from soil were unable to utilise succinate as a carbon source, indicating that the genes were cryptic. Following prolonged incubation on solid media containing succinate as a sole carbon source, two strains (CJ1 and N18) gave rise to succinate-utilising colonies at a frequency much greater than expected by random mutation alone. Sequence analysis revealed that point mutations had occurred within the *dctB* or *dctD* genes in these strains. A cosmid clone pJW5 that conferred the mutator phenotype on other Dct^- non-symbiotic strains was isolated from a CJ1 genomic DNA library. Two gene clusters similar to the toxin-antitoxin module *hipAB,* designated *hipB1A1* and *hipA2B2,* found on pJW5 were implicated in the mutator phenotype. pJW5 was mutagenised with transposon Tn5 and two insertions both within *hipB2* were the only insertions found that abolished the mutator phenotype. Furthermore, it was reported that subclones of pJW5 containing either or both *hipAB* loci conferred the mutator phenotype. These findings led to the hypothesis that the *hipAB* loci enable the cells to mutate to succinate utilisation by allowing them to undergo adaptive mutation through a dormant state known as persistence (Weaver, 2003 and personal communication).

In this study subcloning was employed in an effort to confirm that *hipAB* act as a toxin-antitoxin module and further define the contribution of the *hipAB* loci to the mutator phenotype. Attempts to express *hipA1* or *hipA2* from an inducible promoter failed to show that HipA acted as a toxin. Further subcloning studies suggested that neither individual *hip* genes, *hipB1A1*, *hipA2B2*, or both *hipAB* clusters alone could promote mutation to succinate metabolism, indicating that other gene(s) in addition to *hipB2* present on pJW5 are required. Subsequent restriction digests of pJW5 demarcated a region to the left end of pJW5 as most likely being involved in the mutator phenotype.

The underlying mechanism of mutation to succinate utilisation, although still ambiguous, seems to harbour some functions of stress-induced mutagenesis (adaptive mutation) and cryptic activation of silent genes. Involvement of other genes in the phenotype was postulated and the Bacterial Transcription Repair Coupling Factor (TRCF or Mfd) gene was identified as a possible candidate. Mfd facilitates recruitment of repair proteins to DNA lesions present on the coding DNA strand of actively transcribed genes. An R7ANS *mfd*

mutant was constructed and analysis of the mutant suggested that the *mfd* gene plays a role in the mutator phenotype as the onset of mutant colonies was delayed in the mutant and their numbers were reduced.

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bp	base pair
cfu	colony forming unit
cm	centimetre
ºC	degree celcius
COG	clusters of orthologous groups of proteins
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetra-acetic acid
g	gram
h	hour
IS	insertion sequence
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
min	minute
m	molar
ng	nanogram
nm	nanometer
OD_{420}	optical density at 420
OD_{600}	optical density at 600
ORF	open reading frame
%	percent
p	plasmid designation
ppGpp	guanosine pentaphosphate

RDM	rhizobia defined media
RNA	ribonucleic acid
Rnase	ribonuclease
s	second
Tris	tris(hydroxymethyl) aminomethane
w/v	weight to volume ratio
V	volt

1 Introduction

Mesorhizobium loti is a member of the Rhizobiaceae family and is able to form globular nodules and perform nitrogen fixation on several *Lotus* species. Conjugal transfer of a chromosomally-located symbiosis island from *M. loti* strain R7A allows nodulation of *Lotus corniculatus* by strains that were previously non-symbiotic, hence converting the strains to functional symbionts (Ronson and Sullivan, 1998).

Previous studies in our laboratory have shown that while most non-symbiotic mesorhizobial strains are unable to utilise succinate (Dct^-), two strains display a $Dct^- \rightarrow ^+$ phenotype following prolonged incubation on rhizobium defined medium supplemented with succinate (SRDM) (Weaver 2003, **Figure 1.1**). The $Dct^- \rightarrow ^+$ phenotype is characterised by the appearance of individual colonies after extended incubation (2-3 weeks) that are succinate-positive. The *dctABD* genes responsible for transport of C4-dicarboxylates are present in non-symbiotic mesorhizobia but are cryptic in most strains (Weaver 2003). Introduction of a plasmid that carries the *dctABD* genes of *Rhizobium leguminosarum* into the strains with a Dct^- or $Dct^- \rightarrow ^+$ phenotype converted the strains to a Dct^+ phenotype, confirming that the phenotypes were due to lack of a functional Dct system in the strains.

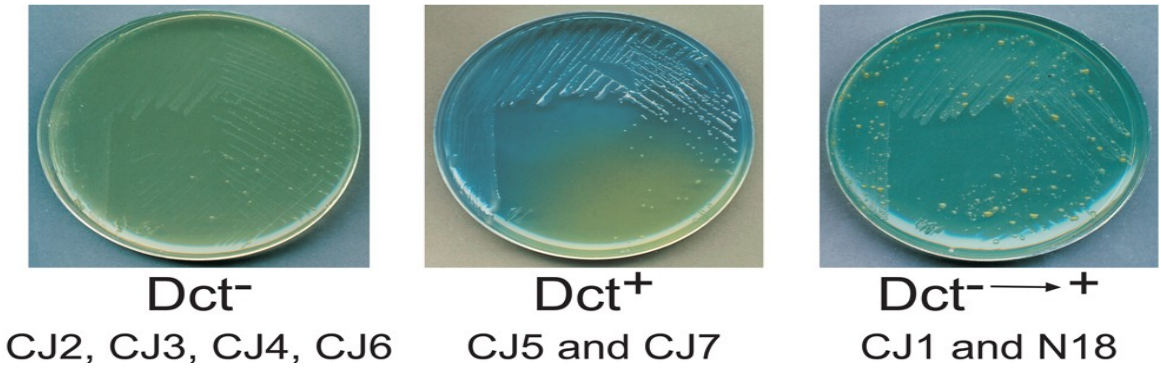


Figure 1.1: Phenotype of non-symbiotic mesorhizobial strains on media with succinate as sole carbon source. Most non-symbiotic strains are unable to utilise succinate (CJ2, CJ3, CJ4 and CJ5) but form microcolonies that metabolise trace carbon sources in the medium (Weaver 2003). CJ5 and CJ7 display a succinate positive phenotype and the pH indicator in the medium turns from green to blue. CJ1 and N18 are initially unable to metabolise succinate but following prolonged incubation (10-14 days), Dct^+ colonies arise.

To identify the genes involved in the Dct$^{- \rightarrow +}$ phenotype displayed by CJ1, a cosmid library of a Dct^{+} derivative of strain CJ1 was constructed and introduced *en masse* into the Dct^{-} strain CJ3. Cosmid pJW5 was isolated from the library and conferred an altered succinate utilization phenotype (Dct$^{- \rightarrow +}$) on CJ3 but did not contain the *dct* genes. Subsequent sequencing confirmed that Dct^{+} colonies of CJ3 containing pJW5 arose through a point mutation in the chromosomal *dctB* or *dctD* genes and that the cosmid was involved in the mutator phenotype.

Transposon Tn5 mutagenesis was used in an attempt to identify genes involved in the mutator phenotype on pJW5. Mutagenised plasmids were transferred to strain CJ3, and an altered Dct phenotype was attributed to insertions within an ORF termed *hipB2*. The *hipB2* mutations delayed the onset of Dct^{+} colonies and substantially reduced but did not eliminate their numbers, suggesting that additional gene(s) were also involved in the mutator phenotype (Weaver 2003). pJW5 contains two loci with similarity to the *hipAB* genes of *Escherichia coli* (**Figure 1.2**). The products of *hipA1* and *hipA2* on pJW5 share 11.5% amino-acid identity while the two *hipB* gene products share 14.6% amino-acid identity (Weaver 2003). The involvement of the *hipAB* genes on pJW5 in the mutator phenotype in non-symbiotic strains was initially confirmed by testing two HindIII and a single PstI restriction fragment subcloned from the cosmid (**Figure 1.2**). Each of the HindIII fragments contained a *hip* locus while the PstI fragment contained both *hip* loci. These cloned fragments were introduced into CJ3 and each conferred a Dct$^{- \rightarrow +}$ phenotype, with the Dct^{+} colonies arising at a similar frequency as with pJW5, indicating that the minimal requirement for the Dct$^{- \rightarrow +}$ phenotype is a single *hip* locus (Weaver 2003 and personal communication).

It has been proposed that the *hipAB* genes constitute a toxin-antitoxin (TA) module in *Escherichia coli,* where *hipA* encodes a toxic factor and *hipB* encodes the antitoxin (Lewis 2005). Expression of the toxin *hipA* has been shown to increase the frequency of cells that are invulnerable to lethal antibiotic doses in model *E. coli* systems (Lewis 2005). This 'persister' state, in most cases, is dependent on the cognate antitoxin *hipB* being intact, highlighting the synergistic nature of this toxin-antitoxin module. These observations led to the hypothesis that the *hipAB* genes allow the bacterial cells under stress to undergo adaptive mutation to metabolise succinate through a state of persistence (Weaver 2003). In addition, previous studies in our laboratory showed that the mutation to Dct^{+} in non-symbiotic strains did not require the recombinase *recA* or involve *relA* that encodes the protein responsible for

producing the alarmone ppGpp. ppGpp is involved in the stringent response in bacteria, causing the inhibition of RNA synthesis when there is a shortage of amino acids present (Magnusson *et al.* 2005).

In this study, I aimed to characterise the *hipAB* operons on pJW5 and in particular how these operons affect mutation to succinate utilisation in *M. loti*. I also studied the effects of the Transcription Repair Coupling Factor (TCRF), a product of the *mfd* gene, as a candidate gene for being involved in the mutator phenotype. The following sections provide the essential background that forms the foundation of this study.

1.1 C4-dicarboxylate transport system (DctABD) of rhizobia

In order for rhizobia to set up an effective nitrogen-fixing symbiosis with a legume plant, the plant must provide the bacteria with a constant flow of energy in the form of reduced carbon compounds (Kahn and Yurgel 2005). C_4-dicarboxylates such as malate, fumarate and succinate are thought to be a major carbon and energy source for the nitrogen-fixing bacteria (Ronson *et al.* 1981). In particular, succinate is a favoured carbon source in many rhizobial genera and in many cases has been shown to exert catabolite repression upon genes needed for the utilization of secondary carbon sources (Bringhurst and Gage 2002). Secondary carbon sources consist of compounds such as glucose, fructose, galactose, lactose, *myo*-inositol, and several pentoses and polyols (Bringhurst and Gage 2002). Succinate has also been shown to be a moderately potent chemoattractant for rhizobia, allowing for coordinated uptake of the metabolite via the dicarboxylate transport system and chemotaxis (Robinson and Bauer 1993).

The dicarboxylate transport system is similar among most members of the Rhizobiaceae family and consists of three genes, *dctA*, *dctB* and *dctD*. The *dctBD* genes comprise a two-component regulatory system, where *dctB* encodes a sensor kinase and *dctD* codes for a response regulator, that activate expression of *dctA* encoding a transmembrane symporter protein DctA (Ronson *et al.* 1987). The membrane-bound histidine kinase DctB senses C4-dicarboxylates through its periplasmic domain and autophosphorylates, transferring a phosphate group onto the response regulator DctD in the process. Phosphorylation activates transcription of *dctA*, the protein product of which transports C4-dicarboxylates into the cytoplasm of the rhizobial cell (Yurgel and Kahn 2004).

As described earlier, non-symbiotic strains of *M. loti* carry a copy of the *dctABD* genes but are unable to utilise succinate as carbon source (Dct^-), indicating that the genes are cryptic.

1.2 Cryptic genes

Cryptic genes are phenotypically silent DNA sequences not normally expressed during the life cycle of an organism but may be activated in a few organisms of a large population by a rare mutational event (Hall *et al*. 1983). The presence of cryptic genes in genomes of a plethora of microbes has raised questions about their role in bacterial evolution (Sankar *et al*. 2009),(Hall *et al*. 1983). In order to further explain the potential benefits of possessing cryptic gene operons, I will focus on the vastly studied cryptic *bgl* operon of *E. coli* (Hall *et al*. 1983) (Sankar *et al*. 2009) (Weaver 2003) (Madan *et al*. 2005) (Madan *et al*. 2008).

The *bgl* operon of *E. coli* is responsible for the uptake and utilisation of aryl-β-glucosides, salicin and arbutin, and is maintained in a silent and uninducible state in wild-type strains (Madan *et al*. 2008). It is similar to the C_4-dicarboxylate transport system of rhizobia in that its regulation is via a two-component regulatory system (Choder and Wright 1993). The *bgl* operon consists of a sensory protein, BglF, present in the cytoplasmic membrane, a regulatory protein, BglG, present in the cytoplasm and a β-glucoside uptake protein BglB (Choder and Wright 1993).

Activation of this otherwise silent operon is through spontaneous mutation, particularly mutations leading to the inactivation of the nucleoid-associated protein H-NS, the function of which, in part, is gene repression and nucleoid structuring (Sankar *et al*. 2009). H-NS independent activation mechanisms exist as well and these include *cis* and *trans* acting mutations and a general state of hypermutability, occasionally leading to the formation of 'adaptive mutants', a concept discussed below (Madan *et al*. 2008) (Hall *et al*. 1983) (Foster 2007). At this point the benefits of increasing mutation rates seem questionable, as many random mutations are seen to be generally deleterious in nature (Foster 2007). Recent studies have shown that strains carrying an activated *bgl* operon exhibit a growth advantage over wild-type, particularly in stationary-phase (Madan *et al*. 2005) (Madan *et al*. 2008). This activation of cryptic genes, under specific conditions, allows the organism to utilise

metabolites during periods of prolonged starvation, a situation similar to the case of the cryptic *dctABD* genes of rhizobia.

1.3 Adaptive Mutation

Many classical experiments like the Luria and Delbrück fluctuation test consolidated Charles Darwin's theory, which states that mutations arise without the influence of selective stress and that environmental stress contributes to the variation upon which natural selection acts (Darwin 1872) (Luria and Delbruck 1943). In 1988, a paper entitled "The Origin of Mutants", published by Cairns and colleagues, suggested that bacteria could choose which mutations to make in response to the environment (Cairns *et al*. 1988). The hypothesis, which contradicts the aforementioned conventional view, demanded a novel phrase for such a phenomenon. 'Adaptive Mutation' seemed appropriate to indicate mutations formed in response to an environment in which the mutations were advantageous (Rosenberg 2001). This theory gained much attention owing to the fact that the classical tests for mutation rates, such as the Luria and Delbrück fluctuation test, utilised lethal selections that were not sensitive enough to detect mutations that arose under selective conditions and demanded that the new mutants grow at the same rate as the parent during the period of the test (Roth *et al*. 2006). The system devised by Cairns and co-workers circumvented this problem by placing the bacterial cells on solid medium and prevented growth by imposing non-lethal stress; new mutants were scored as visible colonies (Cairns *et al*. 1988).

1.3.1 The Cairns system

Following publication of the "Origin of Mutants", Cairns teamed up with Foster to describe the primary experimental evidence for adaptive mutation (Cairns and Foster 1991). They started off with an *Escherichia coli* strain α45, which is unable to utilise lactose as a carbon source but reverts to Lac^+ at a high rate when plated on minimal lactose medium (Foster 2004). The *lac* allele in this strain is a fusion of *lacI* and *lacZ*, which eliminates the *lac* regulatory region and has a +1 frameshift mutation in the *lacI* coding region, carried on a conjugative F plasmid (Foster 2004). Mating the F plasmid from α45 to a recipient *E. coli* strain devoid of the *lacZ* gene and conferring rifampin resistance, resulted in the FC40 strain (Foster 2004). When plated onto minimal lactose medium, the Lac^+ revertants of FC40

accumulate at a constant rate for almost a week, reaching up to 100 Lac^+ colonies per 10^8 cells plated by day 5 (Cairns and Foster 1991).

The mutants only arose when lactose was present, not when they were in a phase of starvation, eliminating the possibility that the appearance of mutants was due to proliferation of the Lac^- progeny (Foster 2004). By locating a second revertible +1 frameshift mutation in the *tetA* gene close to the Lac^- allele in FC40, it was further verified that the mutations were not directed to just the *lacZ* gene (Foster 1997). During selection on lactose, Tet^r revertants appeared at almost the same rate as the Lac^+ mutations, indicating that the two events were not independent and adaptive mutation, as opposed to directed mutation, accounted for this observation.

1.3.2 Mechanisms for Adaptive Mutation

The Cairns system has demonstrated that selective stress in the form of nutrient starvation may activate a mechanism to increase the overall mutation rate. While the likely mechanisms that underlie adaptive mutation are outside the scope of this research, a reasonable awareness of these mechanisms will contribute to a better understanding of this phenomenon.

There are two general mechanisms that are known to occur in the Cairns *lac* frameshift system: adaptive point mutation and adaptive gene amplification (Rosenberg 2001). There have been examples of other systems in bacteria and yeast (Rosenberg 2001) but for the purposes of this thesis, the aforementioned mechanisms will be looked at.

1.3.3 Adaptive Point Mutation

Adaptive point mutation or recombination-dependent mutation states that recombination occurring in non-growing cells stimulates error-prone DNA synthesis (Stumpf *et al*. 2007). This is thought to occur in non-proliferating FC40 cells due to the leaky nature of the mutant *lac* allele, which allows enough energy to be produced to enable replication at the F plasmid's vegetative origin (Foster 2004). Even though the cells are not conjugating, persistent nicking at the conjugal origin would mean that the replication fork will have a high chance of encountering a nick and collapsing, creating a double-strand end and initiating double-strand break repair (Frost and Manchak 1998). RecA catalyses the invasion of a single strand into

the homologous duplex, priming DNA synthesis from the 3' end which requires the actions of the accurate DNA polymerase II (Pol II) or error-prone DNA polymerase IV (Pol IV) (Stumpf *et al*. 2007). If Pol IV overcomes Pol II, it may synthesize a track of error-containing DNA from the *lac* region, giving rise to Lac^+ mutants (Stumpf *et al*. 2007). Consistent with this hypothesis, Pol IV has been shown to be induced in late stationary phase under the control of the sigma factor RpoS (Layton and Foster 2003).

It has been suggested that 10% of the Lac^+ adaptive mutations that are recombination-dependent arise through transient hypermutation (Rosche and Foster 1999.). This proposal stems from the observation that approximately 1% of isolated Lac^+ revertants of FC40 have mutations elsewhere in their genomes, and that these revertants do not have high mutation rates (Torkelson *et al*. 1997.). It is believed that Pol IV is further induced in the hypermutator population, as the extra mutations do not arise if Pol IV is defective (Tompkins *et al*. 2003). In addition, vital components of mismatch repair (MMR) which promotes correction of polymerase errors, have been shown to be downregulated in the hypermutating cells, presumably by the action of RpoS (Layton and Foster 2003).

1.3.4 Adaptive Gene Amplification

In 1998, Andersson and coworkers provided the first suggestion that gene amplification underlies adaptive mutation in the Cairns system (Andersson *et al*. 1998). The system is proposed to work in the following manner: The Lac^- population initially contains a small number of cells that have duplicated the *lac* region and continue to amplify this region due to the leaky nature of the mutant *lac* allele (Hendrickson *et al*. 2002). Amplifying the *lac* region allows the cells to grow slowly on lactose medium producing microcolonies of pseudo-Lac^+ cells. When the total number of *lac* alleles reaches a large enough quantity, the probability of mutation increases, allowing true Lac^+ revertants to occur among the amplified cells. As the Lac^+ revertants multiply, their amplification is resolved so that the Lac^+ cells eventually carry only a single Lac^+ allele (Hendrickson *et al*. 2002).

1.3.5 The Adaptive mutation controversy

We have seen how adaptive mutation can allow for an organism to overcome periods of stress during nutrient deprivation through two generally accepted but mutually exclusive means. There is still, however, much debate over the applicability of the adaptive mutation theory to stress-induced mutagenesis as a whole (Roth *et al*. 2006) (Wrande *et al*. 2008). As previously discussed, adaptive mutation assumes that environmental changes allow bacterial cells to mutate their genomes in order to relieve selective pressure. This is contradictory to the accepted neo-Darwinian theory that has been in place for many years. It also challenges the central dogma theory which states that DNA defines protein expression, and that protein expression creates function in a physical environment (Crick 1970). If the environment provides feedback to DNA to mutate in a given way, such a process would involve an information transfer from the environment to the DNA.

In a review paper entitled, "Stress-Induced Mutagenesis in Bacteria", Patricia Foster provides compelling arguments for the legitimacy of adaptive mutation (Foster 2007). In short, the environment triggers signals that activate various stress responses that enable the overall mutation rate of the bacteria to increase, allowing them to overcome the selective pressure. Although most of these mutations may be deleterious and cause most cells to die, a few cells acquire beneficial mutations that allow growth, resulting in an escape from this high mutation state (hypermutation) and a fixation of the genotype in the population (Hall 1990). Hence adaptive in this context is in *response* to environmental stresses such as nutrient starvation or high doses of lethal antibiotics and not because of it. This view is shared by many other groups including Sung and Yasbin, who have identified an instance of adaptive mutation occurring in *Bacillus subtilis,* a Gram-positive soil microbe (Sung and Yasbin 2002). They have shown in their study that a small population of *B. subtilis* subjected to amino-acid deprivation undergoes a hypermutable state and this subpopulation is involved in adaptive mutagenesis. The regulation of this mutagenesis was further demonstrated to be subject to regulation involving at least two genes required in the regulation of post-exponential growth.

Soil bacteria such as *M. loti* and *B. subtilis* are constantly confronted by stressful growth-limiting and potentially rapidly-changing environments. With the exception of soil directly influenced by plant roots, the soil environment is oligotrophic with carbon availability the

most common factor limiting microbial growth (Kivisaar 2003). Hence the ability of soil bacteria to adapt to changes in available carbon is critical to their evolutionary fitness.

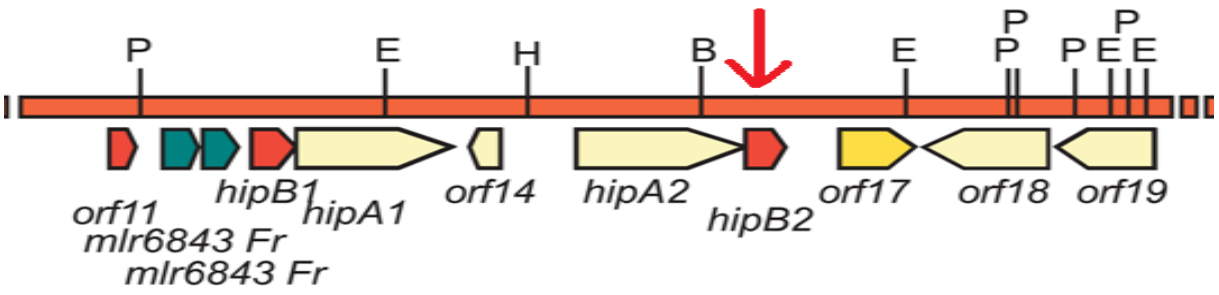
1.4 Bacterial persistence and the role of *hipAB* genes

As discussed, it is proposed that the apparent adaptive mutation phenotype to succinate utilization in strain CJ1 is due to the effects of the *hipAB* genes, which may allow for a state of 'persistence' to be induced, allowing the bacteria to survive high mutation rates. The next few sections describe the *hipAB* genes in greater detail along with the phenomenon of persistence.

Bacterial persistence refers to the ability of a small fraction of cells within a genetically homogenous population to survive challenge by lethal concentration of antibiotics (Korch and Hill 2006). The phenomenon was first described in 1944 by Joseph Bigger, who observed that penicillin did not sterilize a culture of *Staphylococcus* (Keren *et al*. 2004). Persister cells do not have a genetic resistance mechanism, as shown by Bigger who confirmed that the progeny of surviving bacteria were susceptible to penicillin (Keren *et al*. 2004).

In a targeted search for persister genes in the 1980s, Moyed and Bertrand mapped a persister mutation to the *hipA* gene of *E. coli* strain K-12 (Moyed and Bertrand 1983). The mutant was called *hipA7* and exhibited a higher persistence than the wild-type (Lewis 2007). The *hipA7* allele carries two point mutations and confers resistance to many β-lactam and aminoglycoside antibiotics in addition to heat and various DNA-damaging agents (Keren *et al*. 2004).

It has been proposed that the *hipAB* genes constitute a toxin-antitoxin (TA) module where *hipA* encodes a toxic factor and *hipB* encodes the antitoxin (Gerdes *et al*. 2005). This inference stemmed not only from the fact that *hipAB* loci resemble TA modules that are found on plasmids but also that HipB binds HipA in a tight complex to repress its toxicity (Falla and Chopra 1998)(Gerdes *et al*. 2005). **Figure 1.3** shows the location of the *hipB* gene in relation to the *hipA* gene in *E. coli*. **Figure 1.2** depicts the *hipAB/BA* loci in pJW5.



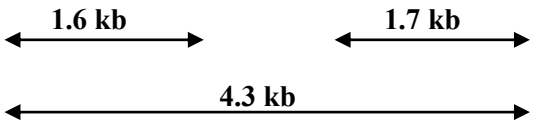


Figure 1.2: pJW5 contains two *hipAB* loci (*hipB1 A1*and *hipA2 hipB2*)**.** The Tn5 insertion in *hipB2* is indicated by the red arrow. P, E, H and B represent the *PstI, EcoRI, HindIII* and *BamHI* restriction sites respectively. Size markers for *hipB1A1* (1.6 kb), *hipA2B2* (1.7 kb) and both *hipAB* operons (4.3 kb) are represented by horizontal arrows.

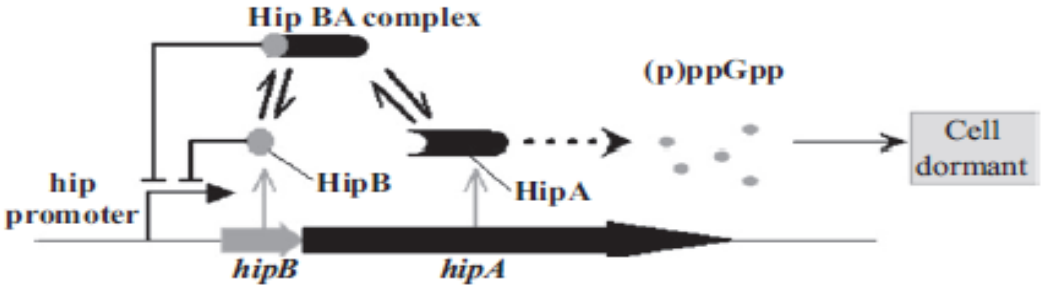


Figure 1.3: A schematic diagram of *hipBA* operons and HipAB functions in *E .coli* (excerpt from (Lou *et al*. 2008)). The grey arrows represent the gene expressions of *hipB* and *hipA*; the dotted arrow highlights an unknown process that requires stress response signals from factors such as (p) ppGpp. HipB can bind tightly to HipA and form a stable non-toxic complex (HipBA). Both HipB and HipBA complex can bind to DNA and repress the transcriptional activity of the *hip* operon (Black *et al*. 1994).

In *E. coli*, the autoregulatory function of the antitoxin *hipB* is vital to the cell as *hipB* mutant strains are non-viable due to the apparent lethality of nonregulated *hipA* expression (Falla and Chopra 1998.). In contrast, the *hipA7* mutated gene has been shown to be non-toxic when

expressed in the absence of *hipB* (Korch *et al*. 2003). One might speculate that the mutant *hipA* allele may confer a greater advantage in non-growing cells than the wild-type, not only because it is less toxic to the cell but also due to the fact that it confers a state of persistence in which a greater number of bacteria are able to survive under stressful conditions. However, a recent paper by Korch and Hill indicates that such a speculation may be inappropriate (Korch and Hill 2006). In their study, Korch and Hill characterised the effects of ectopic overexpression of both wild-type and mutant *hipA* genes on macromolecular synthesis and persister formation in *E. coli*. They found that expressing *hipA* in excess of *hipB* inhibited protein, RNA and DNA synthesis, inducing a transient dormant state (persister state) which was reversible by expression of its cognate antitoxin gene *hipB*. The effects of overexpressing *hipA* did not appear to be bactericidal but bacteriostatic as the colonies were still viable after 72 hours of constant *hipA* induction. The authors proposed that the cells resembled a state whereby they were viable but nonculturable (VNBC). A VNBC state is defined as a reversible, genetically programmed state whereby non-growing cells defend themselves from adverse environmental conditions (Oliver 2005).

In contrast to the wild-type *hipA* allele, *hipA7* overexpression had minor effects on macromolecular synthesis or cell growth but still conferred persistence to a similar percentage of cells. Korch and Hill propose that this observation may be explained in part due to the recessive nature of *hipA7* to the wild-type *hipA* allele. To a greater extent, the high persistence and 'toxin' functions of *hipA* could be separate. This explanation is consistent with previous observations that certain single point mutations inactivate the toxicity of HipA but still confer high persistence (Korch and Hill 2006).

1.5 TA modules in rhizobia

TA modules were initially identified on plasmids acting as post-segregational killing systems. Their function was to prevent the proliferation of plasmid-free progeny or programmed cell-death (Gerdes *et al.* 2005). In further experiments, TA loci were also identified on chromosomes and were considered to be associated with the modulation of the global level of translation under conditions of nutrient limitation, or under various stress conditions (Gerdes *et al*. 2005). Although most TA modules have been charactersied in model systems like *E. coli*, a recent paper has characterized the first functional TA system in *Rhizobiaceae* (Bodogai *et al*. 2006). This system comprises a chromosomal *ntrPR* TA operon in

Sinorhizobium meliloti, encoding an antitioxin (NtrP) and a toxin (NtrR). Discovery of this module first began with the finding that a Tn5 insertion in the *ntrR* gene resulted in increased transcription of *nod* and *nif* genes as compared to that of the wild type strain, and this effect was more pronounced in the presence of an external ammonium source ((Dusha *et al*. 1989); (Oláh *et al*. 2001)). Nodulation (*nod*) genes are involved in the production of the bacterial signal molecules the Nod factors and the *nif* genes encode the nitrogenase enzyme involved in nitrogen fixation (Bodogai *et al*. 2006). Overexpressing the *ntrR* gene under the control of an inducible arabinose promoter in an *E. coli* derivative resulted in the inhibition of cell growth and colony formation, but this effect was counteracted by the presence of the antitoxin NtrP (Bodogai *et al*. 2006). This is typical of TA systems where the antitoxin binds the toxin thus inhibiting its activity. A less effective down-regulation of varied symbiotic and metabolic functions in an *ntrR* mutant under microoxic conditions and an increased symbiotic efficiency with the host plant alfalfa suggest that the *ntrPR* module contributes to adjusting metabolic levels under symbiosis and other stressful conditions (Bodogai *et al*. 2006).

It would hence be of great interest to elucidate the functions of the *hipAB* TA gene loci in assisting microbial evolution. Although their role in adaptive mutation is dubious *per se*, it is clear that the genes facilitate a condition in bacterial cells whereby they are able to increase their mutation rates to overcome stress through the process of persistence.

1.6 The Transcription-repair coupling factor

Proper expression of genes depends on RNA polymerase (RNAP) transcribing the genetic information held in DNA accurately. Mechanisms are in place to allow for repair of damaged DNA where the RNAP has stalled at lesions in the template strand, allowing for transcription to continue and averting any cellular damage (Savery 2007). Transcription-coupled repair is the preferred term for such mechanisms and is carried out by the Transcription Repair Coupling Factor (TCRF), a product of the *mfd* gene (Deaconescu *et al*. 2006). The functions of the *mfd* gene were discovered after *E. coli mfd* mutants showed a reduction in the frequency of nonsense suppressor mutations caused by inhibition of protein synthesis due to mutagenic treatment (Witkin 1966).

The mutants were also sensitive to ultraviolet (UV) radiation and they express stress response functions at lower UV exposure (Deaconescu *et al*. 2006). These mutants are said to be deficient in "mutation frequency decline" (Deaconescu *et al*. 2006).

E. coli TRCF is a 130 kDa protein comprised of 1148 amino acids (**Figure 1.4**). The functions of TRCF is two-fold: (1) It is responsible for relieving transcription-dependant inhibition of nucleotide excision repair (NER) by recognition and ATP-dependant removal of stalled RNAP covering the damaged DNA ; and (2) stimulating DNA repair by recruiting Uvr(A)BC endonucleases (Deaconescu *et al*. 2006).

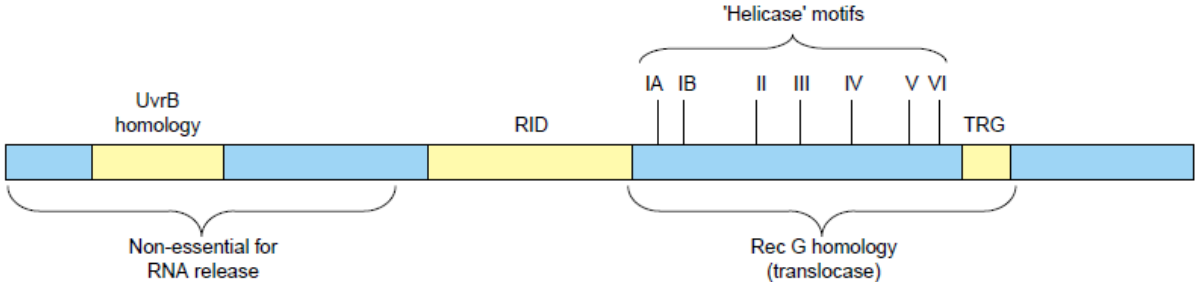


Figure 1.4: Diagrammatical representation of the *E. coli* TRCF protein. The leftmost portion contains a UvrB homologous segment that is believed to act in recruiting the excision repair complex to RNAP but is not required for its release. The RID (RNA polymerase interacting domain) binds TRCF to RNAP, and the 'helicase' motifs along with the TRG (translocase in RecG) element are believed to induce translocation of RNAP along DNA, leading to its release from the template when elongation is prevented. Figure adapted from (Roberts and Park 2004)

1.6.1 TRCF and its role in adaptive mutagenesis

As described above, TRCF plays an important role in ensuring proper transcription of template DNA. Improper repair of DNA can affect both fidelity and processivity of transcription and can threaten the viability of the cell (Savery 2007). Transcriptional mutagenesis, however, may provide a transient growth advantage to the bacterial cell through the production of altered proteins from mutant mRNAs stemming from an unrepaired DNA lesion bypassed by RNAP (Ross *et al*. 2006). Such a system, that harbours functions of TRCF, has recently been shown to induce adaptive mutagenesis in *B. subtilis* by Ross and co-workers (Ross *et al*. 2006). In their study, a *B. subtilis* strain was made *mfd*-deficient by

inserting a tetracycline resistance marker into the gene. They then used this *mfd* mutant strain to carry out stationary-phase mutagenesis studies on reversion of histidine, methionine and leucine auxotrophs to prototrophy. It should be noted that the *mfd*-deficient strains are auxotrophic for these amino-acids before the test and reversion rates were calculated by carrying out fluctuation tests as described in previous studies (Sung and Yasbin 2002). In theory, one would expect that the reversion to prototrophy would increase in the *mfd* mutant strains, as the cellular stress would cause errors in replication and the lack of transcription-coupled repair, would allow for a high level of mutation to be attained and generation of adaptive mutants that can grow on the selected amino acid. In the experiment, however, Ross and colleagues found that loss of *mfd* resulted in a reduction of revertants arising on selective media during the starvation period. A more prominent result was the absence of revertants to leucine prototrophy in this mutant strain. Complementation of the disrupted *mfd* gene in the mutant bacteria with TRCF expressed from an inducible plasmid relieved the defiency in all reversion categories and further showed that TRCF is needed in only minute quantities in stationary-phase cells to support adaptive mutagenesis. Another point of great interest is the fact that a functional *recA* gene was not required for the generation of mutants that confer prototrophy. It would hence be of great interest to elucidate whether the *mfd* homolouge plays a part in mutation to succinate utilisation in non-symbiotic strains of *M. loti*.

1.7 Aims of this study

This study aimed to characterise the functions of the *hipAB/BA* operons located on pJW5 and also elucidate the role of the *mfd* gene in mutation to succinate utilisation in non-symbiotic strains.

Initially, I set out to determine the effects of overexpressing the CJ1 *hipA* gene on adaptive mutation to succinate utilization and cell viability in *M. loti* cells. I then proceeded to clone the *hipA1, hipA2, hipA1B1* and *hipA2B2* genes, and introduce these constructs into CJ3 and R7ANS. This allowed determination of the functions of each gene and the *hip* operon on reversion to succinate utilisation in these strains. Further experiments involving restriction fragments of pJW5, in CJ3 and R7ANS backgrounds, were also carried out to close in on the regions of this plasmid that promote a mutator phenotype.

In the case for *mfd*, I started out by identifying a homologue of the gene in *M. loti* and once determined, proceeded to make a mutant in R7ANS by markerless deletion. Once the mutant was confirmed, it was tested for its effects on succinate utilisation.

2 Materials and Methods

2.1 Bacterial strains and plasmids

Escherichia coli and *Mesorhizobium loti* strains and plasmids used in this study are described in **Table 2.1**

Table 2.1 Strains and plasmids used in this study

Bacterial Strain	Characteristics	Source/Reference
E. coli		
HB101	*leuB thi xyl mtl ara* Δ*(gpt-proA) lacY hsdS recA galK supE mcrB*, Str^R	(Boyer and Roulland-Dussoix 1969)
S17-1/λ*pir*	RK2 *tra* regulon*, pir*, host for *pir*- dependant plasmids	(Miller and Mekalanos 1988)
M. loti		
R7A	Field reisolate of ICMP3153 ; wild-type symbiotic strain	(Sullivan *et al*. 2002)
R7ANS	Non-symbiotic derivative of R7A	(Ramsay *et al*. 2006)
CJ1	Non-symbiotic mutator strain that has a Dct^+ derivative, Nad^+, Bio^-, Thi^-, Dct^-/Dct^+	(Sullivan *et al*. 1996) (Weaver 2003)
CJ3	Non-symbiotic mesorhizobial strain, Nad^-, Bio^-, Thi^-, Dct^-. Does not show mutator phenotype.	(Sullivan *et al*. 1996)
R7ANSΔ*mfd*	*mfd* markerless deletion mutant	This study

Plasmids	Characteristics	Source/Reference
pBluescript II KS	Cloning vector, *ori*ColE1, *lacZα*, ApR	Stratagene
pLAFR1	Broad-host range plasmid, pRK290 derivative with λ *cos* site, *oriT,* IncP1, single EcoRI site. Tc^R.	(Friedman *et al*. 1982)
pIJ3200	pLAFR1 derivative containing the cloning cassette from pBluescript. Tc^R.	(Liu *et al*. 1990)

pJQ200SK	pACYC184-derived (p15a) suicide vector, *sacB*, *oriT*. Gm^R	(Quandt and Hynes 1993)
pProbe	pVS1/p15a derived promoter probe vector, *oriT*. Gm^R	(Miller 2000)
pJW5	Cosmid from *Mesorhizobium* sp. CJ1 Dct^+ library that confers a Dct^- to Dct^+ mutator phenotype on *Mesorhizobium* sp. strain CJ3. Tc^R	(Weaver 2003)
pFAJ1700	Low-copy number, stable broad-host-range plasmid, *oriV*, Tc^R, Ap^R	(Dombrecht *et al*. 2001)
pTH1227	Broad-host-range vector with IPTG inducible expression. Tc^R	(Cheng *et al*. 2007)
pTHUS1	pTH1227 containing the *hipA1* gene from CJ1. Tc^R	This study
pTHUS2	pTH1227 containing the *hipA2* gene from CJ1. Tc^R	This study
pUShipB1A1	*hipA1B1* locus cloned into pFAJ1700	This study
pUShipB1	*hipB1* gene cloned into pFAJ1700	This study
pUShipA2B2	*hipA2B2* locus cloned into pFAJ1700	This study
pUShipA2	*hipA2* gene cloned into pFAJ1700	This study
pUSHindA	HindIII restriction fragment of pJW5 containing *hipB1A1* gene cloned into pFAJ1700	J Weaver, this lab
pUSHindB	HindIII restriction fragment of pJW5 containing *hipA2B2* locus cloned into pFAJ1700	J Weaver, this lab
pUSPstA	PstI restriction fragment of pJW5 containing both *hipB1A1* and *hipA2B2* genes cloned into pIJ3200	J Weaver, this lab
pUSBgl	10 kb BglII fragment of pJW5 containing both *hipB1A1* and *hipA2B2* genes cloned into pProbe.	This study
pUSNhe	NheI self-ligated fragment (pLAFR1 background) of pJW5 that does not contain the *hipAB* operons.	This study
pUSac	SacI self-ligated fragment (pLAFR1 background) of pJW5.	This study

pUSdd	NheI/SacI double digested fragment of pJW5. Self ligated in a pLAFR1 background.	This study
R7ANS /pJW5	R7ANS with cosmid pJW5	This study
CJ3 / pJW5	CJ3 with cosmid pJW5	This study
R7A/*dctA*chr-*gusA*	R7A containing a chromosomal fusion of dctA and gusA in trans. Cloned into pFAJ1700. Serves as positive control for GUS assays	Weaver

2.2 Media and Antibiotics

E. coli strains were cultured at 37ºC on Luria-Bertani (LB) agar or in TY or LB broth (Beringer 1974; Miller 1972). Isopropyl β-D-thiogalactopyranoside (IPTG, 20 μg/mL) was added to agar and broths when required. Mesorhizobial strains were grown at 28ºC on TY or *Rhizobium* defined medium (RDM) agar plates or in TY or RDM broths (Beringer 1974; Ronson *et al*. 1987). RDM was supplemented with either 4% glucose (GRDM) or 20 mM succinate (SRDM) as the sole carbon source. RDM with 5% sucrose as the sole carbon source was used for the selection of non-polar mutants from strains containing pJQ200SK. Media were supplemented with antibiotics as required (**Table 2.2**). Media recipes are given in Appendix A.

Table 2.2 Antibiotics used in this study

Antibiotic	Abbreviation	Concentration (μg/mL)	
		E. coli	*Mesorhizobium* sp.
Ampicillin	Amp	100	-
Gentamicin	Gm	25	50
Kanamycin	Km	50	-
Tetracycline	Tc	15	2
Neomycin	Neo	-	200

2.3 Storage of cultures

Strains to be stored permanently were grown to stationary phase in either LB (*E. coli*) or GRDM (mesorhizobia). Aliquots (800 µL) of culture were mixed with 70 µL of dimethylsulfoxide (DMSO) in Nalgene cryogenic vials (Nalgene®, Rochester, NY, USA) and the vials stored at -70ºC.

2.4 Enzymes and Chemicals

Enzymes were purchased from Roche Diagnostics (Mannheim, Germany) and New England Biolabs (Beverly, MA, USA). Antibiotics were purchased from Sigma-Aldrich (St. Louis, USA), dissolved to an appropriate concentration in distilled water and filter-sterilised through a 0.45 µm syringe filter, with the exception of tetracycline which was dissolved in absolute methanol. All chemicals were analytical grade and solutions were prepared in water unless otherwise indicated. Recipes for solutions and buffers are given in Appendix A.

2.5 Spectroscopy

Optical densities of cultures were determined using a Jenway 6300 spectrophotometer (Jenway Instruments). Absorbance readings and concentrations of DNA were measured using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies).

2.6 DNA isolation and manipulation

2.6.1 Plasmid DNA extraction

2.6.1.1 Boiling minipreps

A boiling miniprep method adapted from (Holmes and Quigley 1981) was used to extract plasmid DNA. *E. coli* cells were grown at 37°C overnight in 5 ml LB broths containing appropriate antibiotics. Culture (1.5 mL) was harvested by centrifugation with pellets then re-suspended in 350 µL HQ-STET buffer (8% [w/v] sucrose, 50 mM EDTA [pH8], 5 mM Tris [pH8], 5% [w/v] Triton X-100). Lysozyme, 25 µL of (10 mg/ mL in 10mM Tris [pH8]), was added before boiling the tubes for 40 s. Immediately following boiling the tubes were

centrifuged at 14, 926 g for 10 min. The gelatinous pellet was removed and discarded. Plasmid DNA was then precipitated from the supernatant via the addition of 450 µL of isopropanol followed by centrifugation at 14, 926 g for 5 min. DNA pellets were then washed in 70% ethanol, air-dried, then re-suspended in 50 µL of filter-sterile Milli-Q water.

2.6.1.2 Alkaline lysis preps

Plasmid DNA was extracted using a method based on Feliciello and Chinali (1993). Overnight cultures (3 mL) grown in LB broths at 37°C were harvested by centrifugation. Cell pellets were re-suspended in 250 µL lysis solution (0.2M NaOH, 1% [w/v] SDS) by vortexing, followed by incubation on ice for 3 min. Neutralising solution (350 µL of 3 M KAc, 5% [w/v] formic acid) was then added and mixed by inversion. The bacterial lysate was then centrifuged at 14,926 g for 5 min and 800 µLof the supernatant transferred to a new tube where the DNA was precipitated by the addition of 560 µL of isopropanol. The samples were centrifuged again at 14, 926 g for 5 min after which the supernatant was discarded and the DNA pellets were washed in 1 mL of 70% ethanol, air-dried and re-suspended in 50 µL of filter-sterile Milli-Q water.

2.6.1.3 Plasmid and cosmid isolation using commercial kits

High quality plasmid DNA was isolated using a QIAGEN® plasmid midi kit (Qiagen, cat # 12162). Cosmid, plasmid and insert DNA for use in subcloning were prepared using a QIAprep® spin miniprep kit (Qiagen, cat # 27106).

2.6.2 Genomic DNA isolation

2.6.2.1 Ultra-quick genomic DNA preparations

Genomic DNA of *Mesorhizobium sp*. strains was isolated by modification of the ultra-quick genomic DNA preparation described by (Gonzalez-y-Merchand *et al*. 1996). TY cultures (5 mL) were grown for 48 h at 28°C with shaking. Cultures (3 mL) was harvested by centrifugation with the bacterial pellets then re-suspended in 500 µL lysis buffer (4 M guanidinium thiocyanate, 1 mM 2-mercaptoethanol, 10 mM EDTA, 0.1% [w/v] Tween-80). The lysate was then snap-frozen in a dry ice/ ethanol bath before incubation at 65°C for 10

min. The snap-freezing/heating process was repeated twice followed by chilling the tubes on ice for 5 min. The lysate was then extracted once in chloroform, once in phenol/chloroform, then once more in choloroform. Genomic DNA was then precipitated using isopropanol, washed in 70% ethanol and re-suspended in 50 µL of filter-sterile Milli-Q water. To aid the re-suspension of DNA, tubes were incubated at 55°C for 1-2 h.

2.6.2.2 PrepManTM Ultra genomic DNA preparations

Crude genomic DNA for use in PCR was extracted from bacterial pellets harvested from 1-mL broth cultures inoculated with single colonies, using 200 µL of PrepMan^{TM} Ultra reagent (Applied Biosystems). Pellets re-suspended in the reagent were boiled for 10 min, ice-cooled for 2 min then centrifuged at 14, 926 g for 3 min. One µL of supernatant was used in PCR reactions.

2.7 Restriction enzyme digests

The restriction enzyme digests were used in DNA cloning and plasmid construct analysis. The plasmid, cosmid and genomic DNA were digested with appropriate enzymes and buffers at 37°C for 2 h. The standard total volume of a digestion was 60 µl, in which filter-sterile Mili-Q water was added to make up the final volume.

2.8 Agarose gel electrophoresis

DNA samples were mixed with bromophenol blue tracking dye (1 mL Milli-Q water, 1 mL glycerol, 1 mL bromophenol blue [10 mg/mL]) prior to loading onto gels made up of 1% agarose dissolved in 1x Tris-acetate (TAE) buffer (40 mM Tris [pH 8], 20 mM acetic acid, 1 mM EDTA [pH8]) containing 1 µg/mL ethidium bromide. Gels were electrophoresed at 90 V for approximately 1 h, or at 22 V overnight. DNA in the gel was then visualised on a UV transilluminator. Gel images were captured using the GDS 5000 Gel Documentation system (Ultraviolet Products Ltd, Cambridge, USA).

2.9 Ethanol Precipitation

Ethanol precipitation was used to remove the restriction enzyme and buffer. An appropriate amount of 3 M sodium acetate (10% of the total volume of the digestion mix) and 150 µL 100% ethanol were added to the digestion mix. The mixture was centrifuged at 13000g for 15 min and the supernatant was removed. Two hundred µL of 70% ethanol was added, followed by centrifugation at 13000g for 5 min, and the ethanol removed. The remaining pellet was left to air dry for 10-15 min, and then suspended in an appropriate volume of filter- sterile MilliQ water.

2.10 Primer design

The primers were manually designed from the gene sequences. The following criteria were applied when designing primers; 50% GC content, no complementary base pairing, no repeats of more than 3 bp of each nucleotide base and the last 5 bp at 3' end contain only 2 Gs or Cs. The software program Vector NTI (Invitrogen Life Technologies) was used to confirm that the primer sequence would not bind to any undesirable region in the genome. The sequences of PCR primers used in this study are listed in **Table 2.3**. Primers were ordered from Invitrogen Life Technologies.

2.11 Polymerase Chain Reaction (PCR)

PCR was used for many purposes including amplification of DNA for mutant construction, strain confirmation and sequencing. All PCR cycling was performed using a Hybaid PCR express thermal cycler (Hybaid Ltd).

2.11.1 Standard PCR protocols

PCR reactions used the Phusion High-Fidelity PCR system (Finnzymes). Standard 100-µL PCR reactions typically contained 20 µL 5 X Phusion HF buffer, 3 µL DMSO, 2 µL of supplied dNTPs (10 mM), 1 µL of each primer (final concentration 0.5 µM), 1 µL of

template, filter-sterile Milli-Q water to make the volume 100 µL and finally 0.5 µL of Phusion DNA polymerase enzyme.

The following Phusion PCR programme was used:

1 cycle: 98°C (1 min)

25-30 cycles: 98°C (10 s) 57°C* (10-30 s) 72°C (15-30 s/ 1 kb product)

1 cycle: 98°C (10 s) 57°C* (10-30 s) 72°C (5 min)

*Temperature varied dependant on primers.

Table 2.3 PCR primers used in this study

Primer name	Sequence (5'-3')[a]	Used For
CJ1 hipA1 left	AGATCTGGGAGATTTTCATG CCTGACTGGCACGCCCA	5' primer for amplifying *hipA1*
CJ1 hipA1 right	CTGCAGCGGCCGTTCCCCAG CGTTAT	3' primer for amplifying *hipA1*
CJ1 hipA2 left	AGATCTGGGAGATTTTCATGA AACTTACTATTGAGACA	5' primer for amplifying *hipA2*
CJ1 hipA2 right	CTGCAGCATCTTGGCATCGTC GTCTG	3' primer for amplifying *hipA2*
hipA25crseq	CATATGTTCTGCGAGCTTTC	Obtaining start sequence of *hipA2*
hipA2900FWDSEQ	ACGAGGACTATATCGATGCT	Amplifying internal region of *hipA2*
HIPA1800CRSEQ	GAAATCGATGATCTCTGTAT	Amplifying internal region of *hipA1*

HIPA1360SEQCR	GGTATCATCGGACAGCGTCA	Obtaining start sequence of *hipA1*
JWA-1	AAACGTCAGGCTGGCACTGG	Amplifying region surrounding *msi169*
EcoB-2(#2)	GGTAGCAAGCTGCATTGGAG	Amplifying region surrounding *msi169*
EcoB-3	GAATGCCCACGGTGATGTCA	Amplifying region surrounding *msi169*
hipA2asp3prime	AAATTTGGTACCCGTCGTCT GCCTTATGTAG	3' primer for amplifying *hipA2*
hipB1asp3prime	AAATTTGGTACCCAGTCAGT CAGGCATCAGAATGAC	3' primer for amplifying *hipB1*
hipB1asp5prime	TTTAAAGGTACCGCGTCGAT GTCTATTTCGAA	5' primer for amplifying *hipB1*
hipA1asp3prime	AAATTTGGTACCCCCAGCGT TATACGTCATAT	3' primer for amplifying *hipA1*
hipb2asp3prime	AAATTTGGTACCTCACGCGG CCACATTTCAAC	3' primer for amplifying *hipB2*
hipA2asp5prime	TTTAAAGGTACCGTGGCGACA GAAGAATCGCT	5' primer for amplifying *hipA2*
hipB1internalseq 5prime	TGTGGCCTGATAGCGCAATA	Sequencing

hipA1B1internal seq3prime	TAGATGCCACCGAAAGGTGG	Sequencing
hipA2B2internal seq5prime	GTCAGAGCGCCTTCCTCGAA	Sequencing
hipA2internalseq 3prime	TGTGATCTCGTCGGAAGACG	Sequencing
hipA2B2internal seq3prime	AGTGCATGGCCTGCCTGAAA	Sequencing
hipB1internalseq 3prime	TGCCAGTCAGGCATCAGAAT	Sequencing
TCRFseqRR	TCGAACGGCCGCGTCTTGTA	Amplifying right flanking arm of MFD
TCRFseqRL	TTTCCGCAAGCGCGAGTTCT	Amplifying right flanking arm of MFD
tcrfRLextend	GCAGATGTCGGTCGTGCTGA	Amplifying right flanking arm of MFD
TCRFseqLRNEW	ACGCATTGGCGGTGGTGAGA	Amplifying left flanking arm of MFD
TCRFseqLLNEW	TTCTCGACGATGCGGCCGTC	Amplifying left flanking arm of MFD
R7AMFDRRspeI	TTTAAACTAGTCGGTCGCCG ATCCAGATCAT	Markerless Deletion Mutagenesis
R7AmfdLLapaI	AAATTTGGGCCCATGAAGGC GACGTCCTGCCA	Markerless Deletion Mutagenesis

R7AmfdOELR	AATGGTCCTCAGGCCGCCTTC GACACCGTCGACGATGAAC	Overlap-Extension PCR
R7AmfdOERL	GTTCATCGTCGACGGTGTCGA AGGCGGCCTGAGGACCATT	Overlap-Extension PCR
CJ3mfdNEWFWD	TTTAAAGGTACCAAGGACGCA TGATGTCAGCT	Insertional Duplication Mutagenesis
CJ3mfdNEWREV	AAATTTGGTACCGGTGCTGAG TGCGTTGAGAT	Insertional Duplication Mutagenesis
mfdFORW250	CATGCGCGAGATGGATCTTA	Sequencing
mfdREV250	GAACCTGCTTGATCGGCTTT	Sequencing
25mer5primeTCRF	CATCGCCCTGCCAGCAGCCGC AGCAT	Amplify internal portion of mfd
25mer3primeTCRF	TAGGGCAGACAGTCCCAGGCC GGCA	Amplify internal portion of mfd
mfd5primeSUI1	CGGCGTTCGAAGCGCAAGAA	Sequencing
mfd5primeSUI2	ATCGTCGTTCATGCCGACCA	Sequencing
mfdCRsui5prime NEW	AGTGCTGGCGCGACAACAAT	Sequencing
mfdCRsuiNEW	AAACGCGCGGCAAACTCGTC	Sequencing

bamfdcj3REV	TTTAAAGGATCCGATCAGCCG ATCCATTGGCT	Insertional Duplication Mutagenesis
pstmfdcjpfusFWD	AAATTTCTGCAGGCCGCATGA TTGCCTTGAGA	Insertional Duplication Mutagenesis

a Restriction sites incorporated into primers are underlined

2.12 Subcloning of DNA fragments

2.12.1 Preparation of insert and vector DNA

Purified insert DNA and vector DNA were digested with the desired restriction enzyme(s). Following digestion, vectors were dephosphorylated by the addition of 2 µL alkaline phosphatase (Catalog no. 713 023, Roche) and incubated at 37ºC for 30 min. Both insert and vector DNA were then gel-extracted and purified using the QIAEX-II gel extraction kit (QIAGEN) according to the manufacturer's instructions.

2.12.2 Ligations

Appropriate volumes of linearised vector and insert DNA molecules were mixed with 2.5 µL of 10× ligation buffer and 1.5 µL of T4 DNA ligase (Roche) in a total volume of 20 µL, then incubated overnight at 12ºC. Ligated DNA was ethanol precipitated and re-suspended in 5 µL of filter-sterile Milli-Q water.

2.13 Preparation of electrocompetent cells

2.13.1 *Escherichia coli*

Electrocompetent cell stocks of *E. coli* strains HB101 and S17-1λ *pir* were prepared using a protocol adapted from (Sheng *et al*. 1995). Strains were grown to stationary phase in 5-ml LB broths and 0.5 mL of this culture was used to inoculate 500 mL broths that were incubated at

37ºC with shaking at 200 rpm. Cultures were harvested at OD_{600} by centrifugation at 4ºC for 10 min at 5,930 g. Following two washes in 500 mL chilled 10% [w/v] glycerol, cells were washed in 30 mL of chilled 10% glycerol, pelleted, then re-suspended in 1 mL of 10% glycerol, aliquotted into 40 µL volumes, then snap-frozen in dry ice/ ethanol baths. Cells were stored at -80ºC.

2.13.2 *Mesorhizobium spp.*

Electrocompetent *Mesorhizobium* cells were prepared by a method developed by Kazuhiko Saeki (John Sullivan, personal communication). Five mL TY broth cultures of strains were grown to stationary phase. One mL of the culture was the used to inoculate 200 mL TY broths which were incubated at 28ºC with shaking. Cells were harvested at OD_{600} 0.1-0.3 by centrifugation at 4ºC for 10 min at 5,930g. Pelleted cells were washed in 200 mL chilled 10% glycerol, centrifuged, washed in 100 mL 10% glycerol, centrifuged, washed in 4 mL 10% glycerol, centrifuged, then re-suspended in 200 µL 10% glycerol. Forty µL aliquots were snap-frozen in a dry ice/ ethanol bath and stored at -80ºC.

2.14 Electroporation

Electrocompetent cells (40 µL) were thawed on ice. DNA (1-2 µL) was added to the thawed cells before the cells were transferred to a pre-chilled 1 mm gapped electroporation cuvette (Biorad). Cells were then transformed at 1500 V for *E. coli* strains and 1800 V for *M. loti* using an *E. coli* TransPorator electroporation system (BTX). Immediately following shocking, *E. coli* cells were suspended in 1 mL of LB broth and incubated at 37ºC with shaking for 45 min. *Mesorhizobium* cells were suspended in 1 mL of TY broth and incubated at 28ºC with shaking for 4 h. Following incubation, aliquots were spread onto media containing appropriate antibiotics.

2.15 DNA sequencing

High-quality template DNA to be sequenced was prepared using QIAGEN miniprep or PCR purification kits. Relevant primers were mixed with the template DNA and filter-sterile Milli-

Q water, and the mixture sent to the Allan Wilson Centre Genome Service (Massey University, Palmerston North or Albany, NZ) for sequencing.

2.15.1 Computer analysis of DNA sequence data

DNA sequences were viewed, edited and assembled into contigs using the computer program Vector NTI Advance 10 (Invitrogen). National Centre for Biotechnology Information (NCBI) databases were searched for similar nucleotide or amino acid sequences using Blast N, X and P (Altschul *et al*. 1997). Comparisons of sequence data to *M. loti* MAFF303099 and *M. loti* strains R7ANS or CJ3 were carried out using Rhizobase website. (http://genome.kazusa.or.jp/rhizobase).

2.16 Spot matings

2.16.1 Bi-parental spot matings

Introduction of plasmids from *E. coli* strain S17 into *M. loti* R7ANS was achieved through bi-parental spot matings. *E. coli* strain S17 has an integrated plasmid containing *trb* genes, which encode for proteins involved in the formation of a conjugal bridge. The formation of a conjugal bridge allows for the transfer of the DNA from a donor to recipient cell.

The donor *E. coli* and recipient *M. loti* strains were grown to stationary phase in TY broth. Aliquots of each culture (30 µL), were dispensed together as a spot onto an TY agar plate and incubated at 28°C overnight. The resultant bacterial growth was then streaked onto selective medium containing appropriate antibiotics.

2.17 Mutagenesis

Primer pairs were designed to amplify 1 kb left and right arm PCR products that represented flanking regions of target genes. Primer design incorporated SpeI restriction enzyme sites at the outermost end of the left and right arms as well as overlapping sequence to allow the arms to join in-frame. Overlap-extension PCR was employed to amplify 2 kb fragments consisting

of the left and right arms joined in-frame by overlapping sequence which were then ligated into SpeI-digested pJQ200SK. The recombinant plasmid was electroporated into *E. coli* S17 cells then mated into *M. loti* R7ANS via bi-parental spot-matings. Colonies exhibiting Gm^R, provided by pJQ200SK, were passaged for single colonies and confirmed by PCR. A second crossover event to remove pJQ200SK and the wild-type copy of the target gene was forced by plating single crossover clones on RDM containing 5% sucrose. The plasmid pJQ200SK encodes *sacB*, the product of which is lethal in the presence of sucrose. PCR was used to confirm second-crossover clones and the resultant markerless deletion mutants.

2.18 Liquid GUS assays

Seeder broths of strains to be assayed were grown in 5 mL TY or GRDM broths at 28° C for three days. Fifty µL, 100 µL and 200 µL inocula of the cultures with 0.1-5 mM IPTG and pTH1227 with 0.1 – 5.0 mM IPTG were grown overnight at 28°C to mid-log phase. The $OD_{600\ nm}$ of the overnight cultures was measured and broths at mid-log phase ($OD_{600\ nm}$ = 0.3-0.7) were centrifuged to pellet the cells and resuspended in 0.9% saline to give an $OD_{600\ nm}$ around 0.6. GUS buffer (50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 0.1% Triton X-100, and 1 mM p-nitrophenyl β-D-glucuronide (PNPG)) was added to all reaction tubes and made up to 990 µl where needed. Toluene was added to all the tubes to permeabilise the cell membrane and the tubes vortexed for 10 s. Reactions were started by addition of PNPG and placed in 37°C waterbath until the reaction tubes showed visible colour change and ended by aliquoting 200 µl of reaction mixture to 700 µl of 0.46 M sodium carbonate. The $OD_{405\ nm}$ of the reaction mixture was then recorded. The amount of PNPG hydrolysed per minute was then calculated via the equation in GUS protocols, in Miller units (Wilson *et al*. 1992).

Miller units = $OD_{405\ nm}$/ t (min) × V (volume of reaction mixture in mL) × $OD_{600\ nm}$ × 1000

3 Results

3.1 Effects of overexpressing the *hipA1* and *hipA2* TA module on adaptive mutation to succinate utilization and cell viability in *Mesorhizobium loti*

The aim of the experiments described in this section was to achieve controlled expression of *hipA1* and *hipA2* from CJ1, To achieve this, the genes were cloned into an inducible expression vector pTH1227 (**Figure 3.1**). The vector is a pMP220 derivative which has polylinker sites and a promoterless *gus* reporter gene downstream of a Ptac promoter which is inducible by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Cheng *et al*. 2007).

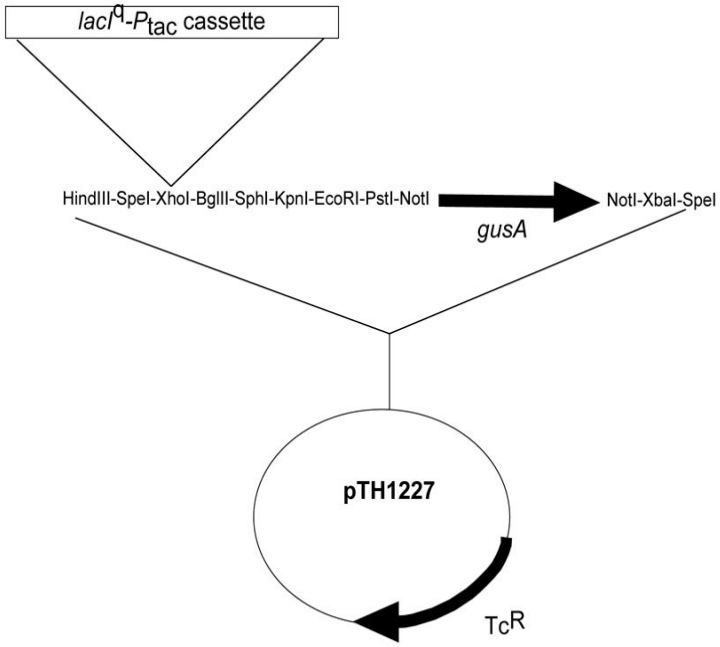


Figure 3.1: Diagram of vector pTH1227. The plasmid carries a tetracycline resistance marker and a reporter *gusA* gene downstream of the Ptac promoter. The vector contains multiple cloning sites that facilitate insertion of a cloned segment. (Map courtesy of Julie Weaver)

3.1.1 Cloning *hipA1* and *hipA2* into pTH1227

The *hipA1* and *hipA2* genes were amplified by PCR (**Figure 3.2**) from CJ1, using primers CJ1 hipA1 left, CJ1 hipA1 right, CJ1 hipA2 left and CJ1 hipA2 right (**Table 2.3**)**.** Since the genes were not expressed from their native promoter, primers were designed to allow for 20-30 bp upstream and downstream of each gene being amplified. The vector and the amplified *hipA1* or *hipA2* gene region were then digested with BglII and ligated.

Plasmid DNA was isolated from 4 transformants and digested with BglII enzyme to identify clones with inserts of the expected size. To confirm that the genes had been cloned into the vector successfully, plasmid DNA was sequenced with hipA25crseq, hipA2900FWDSEQ, HIPA1800CRSEQ and HIPA1360SEQCR primers (**Table 2.3).** The sequences of the cloned *hipA* genes were determined to be devoid of any mutations, and so plasmids pTHUS1 and pTHUS2, harbouring the *hip* genes in pTH1227, were introduced into R7ANS by electroporation.

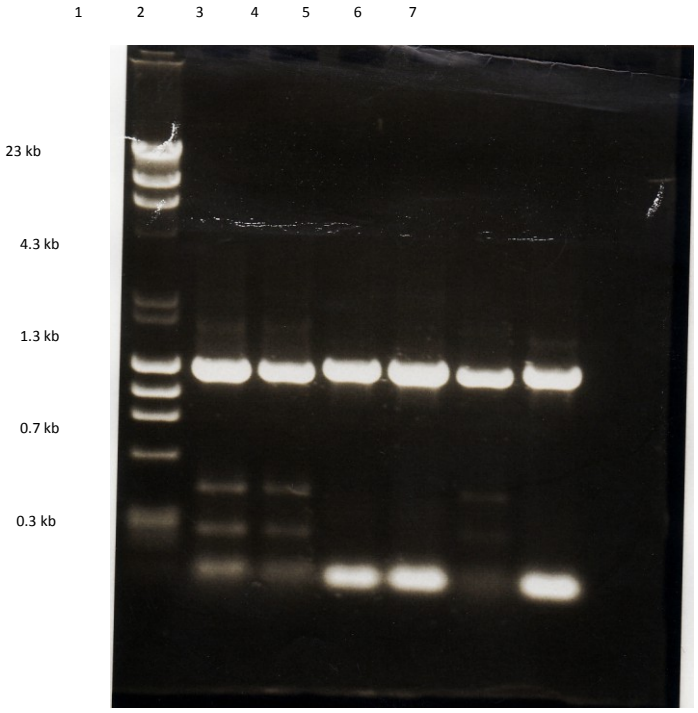


Figure 3.2: Agarose gel showing PCR products amplified from CJ1 or pJW5. Lane 1, λHindIII plus ΦX174 DNA Hae III marker. Lanes 2 and 3, *hipA1* PCR product from CJ1. Lanes 4 and 5, *hipA2* PCR product from CJ1. Lane 6, pJW5 with *hipA1* specific PCR primers. Lane 7, pJW5 with *hipA2* specific PCR primers. Lanes 5 and 6 serve to act as a control to ensure products from CJ1 are identical in size to those amplified from pJW5.

3.1.2 Liquid GUS assay on *hipA1/2* constructs.

The purpose of this assay is to analyse the expression of *hipA* genes under control of the Ptac promoter. It is based on determining the activity of β-glucuronidase, an enzyme from *E. coli* that can form coloured products when incubated with specific substrates such as 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic (X-Gluc) and PNPG (Jefferson *et al*. 1986). The positive control for the GUS assays was R7A/*dctA*chr*gusA*.

The bacterial cultures for the assay were grown in two different broth media, TY and GRDM. This was done to ensure that the complex growth medium (TY) did not contribute to any trace amounts of lactose that could distort the final results by interacting with the *lac* promoter. The concentration of IPTG used for induction of bacterial cultures ranged from 0 to 5.0 mM. R7ANS was used as the negative control and strain R7ANS containing vector pTH1227 alone was assayed in addition to *hipA1* and *hipA2* to demonstrate the activity of the promoter region. The substrate for GUS used was PNPG, which produces a yellow-coloured product when hydrolyzed.

It was observed that the promoter region was very active as the basal levels of activity without IPTG reached approximately 170 units of expression (**Figure 3.3**) as compared to about 20 units of expression for the positive control, R7A/*dctA*chr*gusA*. Induction with more than 1.0 mM IPTG showed a general decline in activity of the promoter region suggesting that induction of the promoter was reaching saturation point. The subsequent concentrations of IPTG used in further experiments were hence 0, 0.1 or 1.0 mM. This range gave induction from near minimum and near maximum expression from the promoter region.

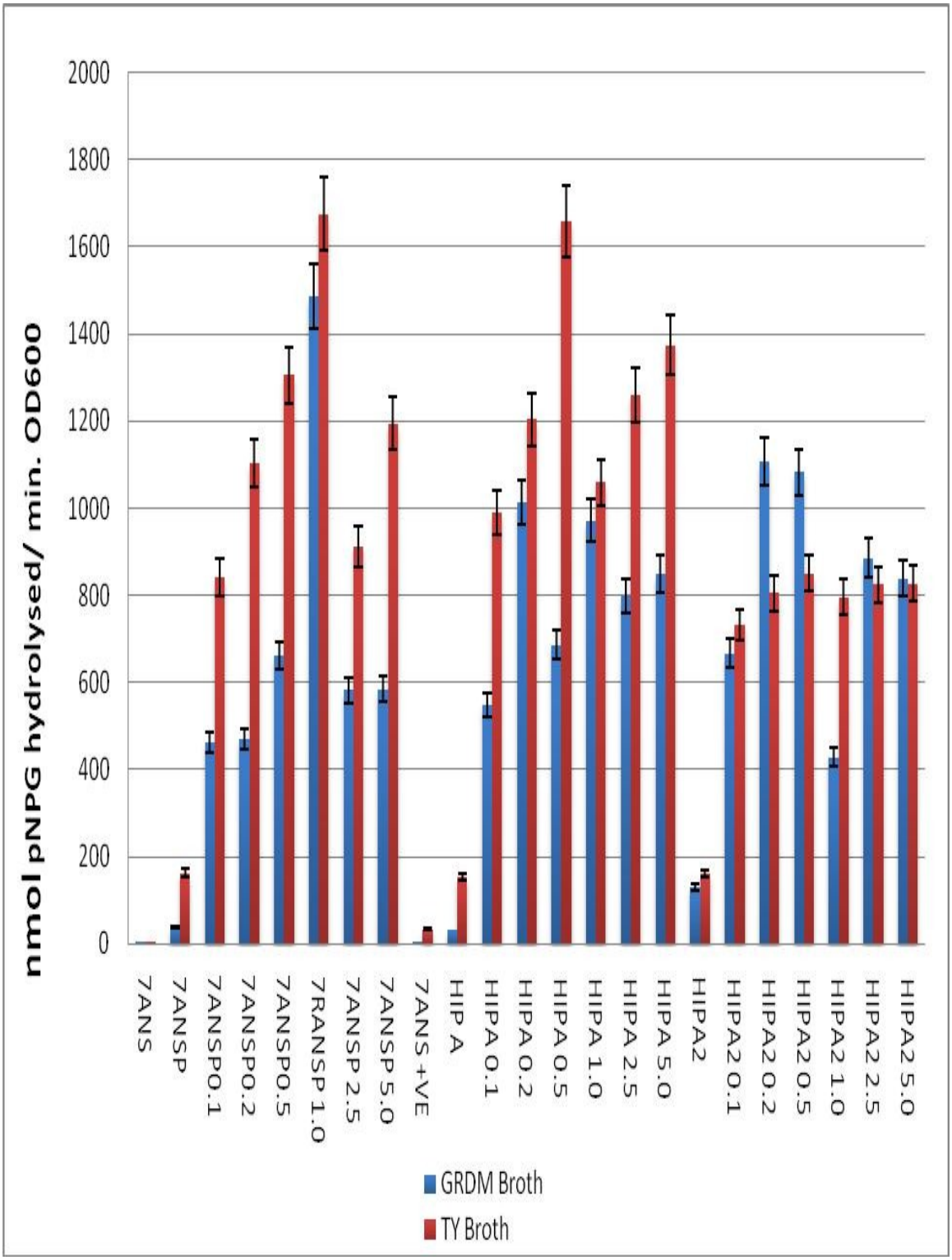


Figure 3.3: GUS activity assay of constructs containing *hipA1* and *hipA2*. Strain R7A containing the vector without an insert is labelled as R7ANSP and the succeeding numbers quantify concentration of IPTG added. Similarly, HIPA and HIPA2 label for R7A containing plasmid name (*hipA1*) and plasmid name (*hipA2*) respectively. The activity of the promoter is reflected on the y-axis as the amount of substrate (PNPG) hydrolysed per minute per OD_{600} unit of cells. Error bars are also shown on the graph.

3.1.3 Colonies on SRDM show unusual Dct+ phenotype

Serial dilutions of R7ANS cultures containing pTHUS1 and pTHUS2 along with the vector alone were plated onto succinate medium and succinate medium supplemented with either 0.1 or 1.0 mM IPTG. R7ANS containing pJW5 and R7ANS without a plasmid served as a positive and negative control respectively. The cultures were grown in TY broths for 48 h and dilutions ranged from 10^{-4} to10^{-8}. Data for these dilutions are given in **Table 3.1** and corresponding graphs in **Figure 3.4**. No impact of plasmid or the presence of IPTG on growth rate or viability was observed.

Table 3.1: Total number of colonies and total number of putative Dct^+ mutants seen 2-3 weeks post plating with respective strains when plated onto SRDM. Dilutions ranged from 10^{-4} to 10^{-8} dilution data omitted as no colonies were observed for all strains.

Total number of colonies/Total number of mutants				
Strain	Dilution/10^			
	-4	-5	-6	-7
R7ANS	TMTC/0	350/0	145/0	18/0
R7ANS/pTH1227	TMTC/0	328/0	123/0	13/0
R7ANS/pTH1227 0.1 mM IPTG	TMTC/0	339/5	127/4	13/4
R7ANS/pTH1227 1.0mM IPTG	TMTC/0	348/7	133/4	22/4
R7ANS/pTHUS1	TMTC/0	320/0	123/0	19/0
R7ANS/pTHUS1 0.1 mM IPTG	TMTC/0	298/8	121/5	17/5
R7ANS/pTHUS1 1.0 mM IPTG	TMTC/0	319/7	119/5	17/6
R7ANS/pTHUS2	TMTC/0	311/0	120/0	14/0
R7ANS/pTHUS2 0.1mM IPTG	TMTC/0	328/8	134/6	14/5
R7ANS/pTHUS2 1.0 mM IPTG	TMTC/0	328/7	112/6	15/6
R7ANS/pJW5	TMTC/144	329/93	122/67	21/14

By three weeks after incubation at 28^{o}C, R7ANS/pJW5 plates showed mutation to succinate utilization (**Figure 3.5**). At this time neither R7ANS nor the R7ANS/pTH1227 or pTHUS1/ pTHUS2 plates in the absence of IPTG showed mutants. After five weeks incubation, the R7A/pTHUS1 and pTHUS2 plates at 10^{-5}, 10^{-6} and 10^{-7} dilutions formed a similar number of mutant-like colonies (data not shown) that were mucoid and shiny in appearance, very much similar to those seen on R7ANS/pJW5 (**Figure 3.6**). Such colonies were observed on plates containing 0.1 and 1.0 mM IPTG but not plates without IPTG. These colonies however failed to show all signs of succinate utilisation as the pH indicator present in the medium did not turn blue. No Dct^+ colonies were observed on the 10^{-4} dilution plates which contained a lawn of cells rather than distinct single colonies.

Surprisingly, the strain containing only the vector gave rise to apparent mutants after 6 weeks incubation (**Figure 3.7**). The 'revertant' colonies from R7ANS/pTHUS1, R7ANS/pTHUS2 and the vector plates were single colony purified onto SRDM and they retained the Dct^+ phenotype indicating that they represented true mutation events (**Figure 3.8**). This result, however, does not reflect on the ability of these strains to allow for a mutator phenotype as is highlighted in **Figure 3.4.** The number of mutants from strains harbouring pJW5 far exceeded the basal levels observed in these test strains. Furthermore expression of all strains harbouring pTH1227 was driven by the Ptac promoter and this could have had an effect on the final result. In addition, the involvement of the *hipB* antitoxin on the mutator phenotype was not taken into consideration. I hence decided to carry out further investigation with the *hipA* and *hipB* genes under the control of their native promoter.

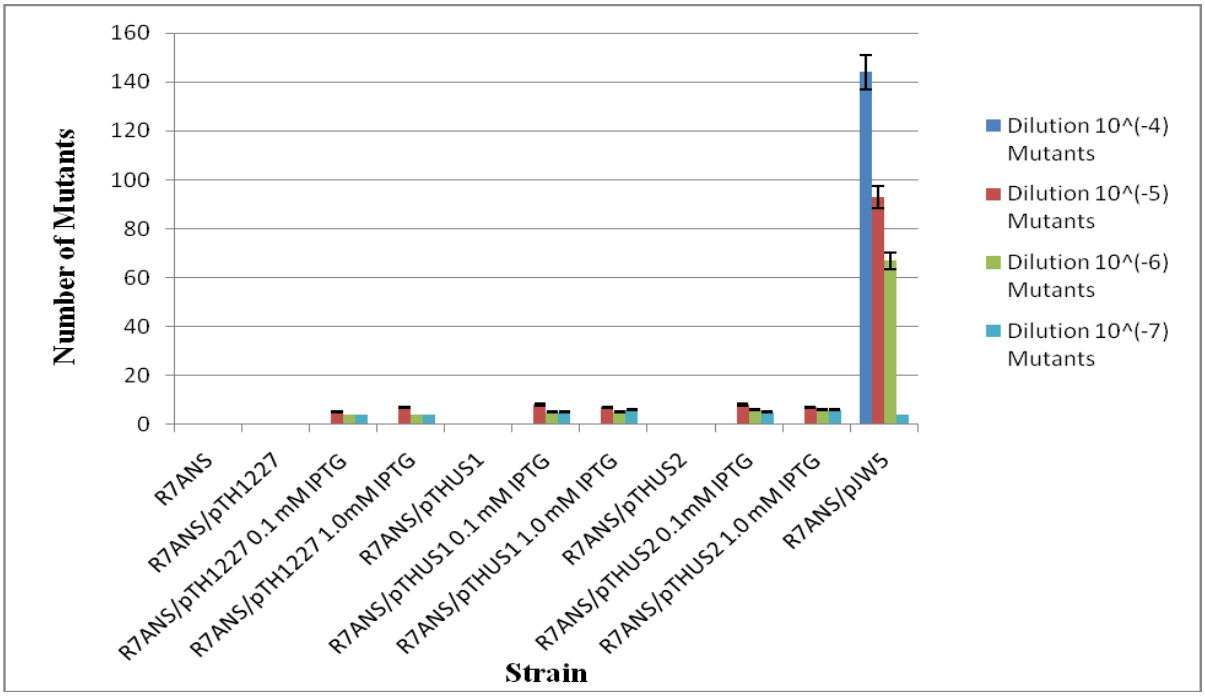


Figure 3.4: Histogram representing total number mutants for the corresponding strains at varying dilutions. The numbers of colonies and revertants are listed in **Table 3.1**. Error bars are also shown on the graphs.

Figure 3.5 Appearance of putative Dct^{+} mutant colonies on R7ANS/pJW5 plate 3 weeks post inoculation at 10^{-5} dilution.

A

B

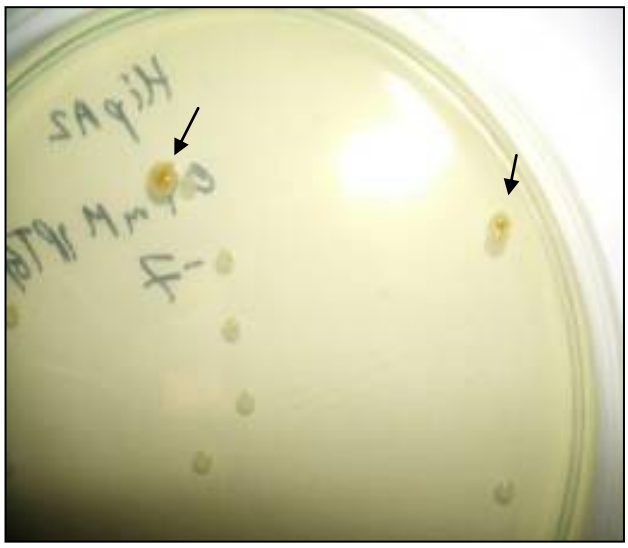


Figure 3.6: **A** Appearance of putative Dct^{+} mutant colonies on R7ANS/pTHUS1. **B** R7ANS/pTHUS2, 5 weeks post inoculation, indicated by the arrows. Both plates at 10^{-7} dilution and 0.1 mM IPTG concentration.

Figure 3.7: Appearance of putative Dct$^+$ mutant-like colonies on vector only plates, 6 weeks post inoculation. Plate shown is the vector on 0.1 mM concentration IPTG and 10^{-5} dilution.

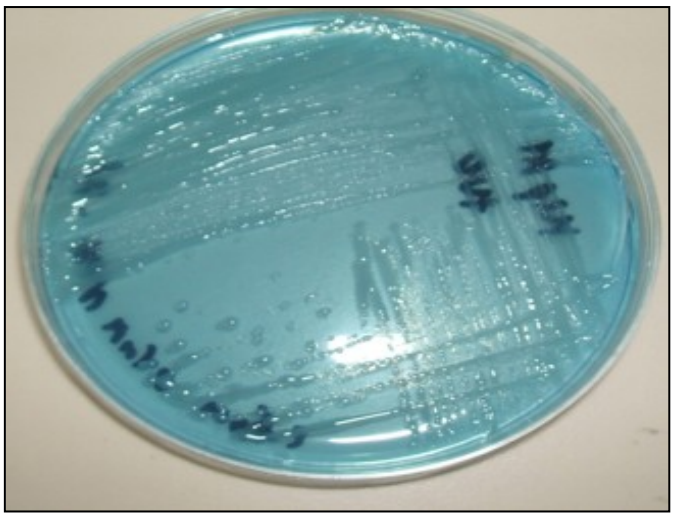


Figure 3.8: Plate of revertant HipA1 colony from **Figure 3.6** on succinate medium. HipA2 and vector colonies showed a similar result on the medium indicating utilisation of succinate.

3.2 Effects of cloning the *hipAB* operon into rhizobia

The results from the expression studies did not show any significant effect of the *hipA1* and *hipA2* genes. This prompted further study to characterise the *hipAB* operon with its native promoter in both R7ANS (which has a very low background reversion to Dct$^+$) and CJ3 (strictly Dct$^-$ phenotype).

To characterise the *hipAB* operon in rhizobia, four regions were amplified from pJW5 (**Figure 3.9**) and ligated into plasmid pFAJ1700. The four regions were as follows:

1. *hipA2B2* – amplified using primers hipA2asp5prime and hipb2asp3prime
2. *hipA2*– amplified using primers hipb2asp3prime and hipA2asp3prime
3. *hipB1*– amplified using primers hipB1asp5prime and hipB1asp3prime
4. *hipB1A1*– amplified using primers hipB1asp5prime and hipA1asp3prime

The products were designed to ensure that the genes were preceded by generous putative promoter regions encompassing the intergenic regions upstream of each gene (366 bp preceding *hipB1* and 585 bp preceding *hipA2*). The constructs were then electroporated into *E. coli* S17 cells, plated, and alkaline lysis preps of plasmid DNA were prepared. These were then digested with BglII to confirm the presence of the expected inserts. PCR was then performed with vector-specific primers to amplify the insert regions for sequencing (**Figure 3.10** and **Figure 3.11**). Once the sequences of the inserts were confirmed, the four plasmid constructs were electroporated into both CJ3 and R7ANS and plated on G/RDM plates.

To elucidate the effects of *hipAB* on the succinate reversion phenotype, serial dilutions (from 10^{-3} to 10^{-7}) of the strains containing the four constructs and complementation clones (these are restriction fragments from pJW5 that were able to impart the mutator phenotype on CJ3, namely: pJWHindA, pJWHindB and pJWPstA (personal communication- Julie Weaver) (**Figure 3.9)** were spread plated onto succinate media (with tetracycline where appropriate). In addition, CJ3/pJW5 and R7ANS/pJW5 were spread plated in parallel as positive controls. The negative controls were CJ3/pFAJ1700 and R7ANS/pFAJ1700. Strains plated numbers of colonies and number of revertants are given in **Table 3.2** and **Table 3.3**. Again, no effect of the plasmids on viability or growth rate was observed.

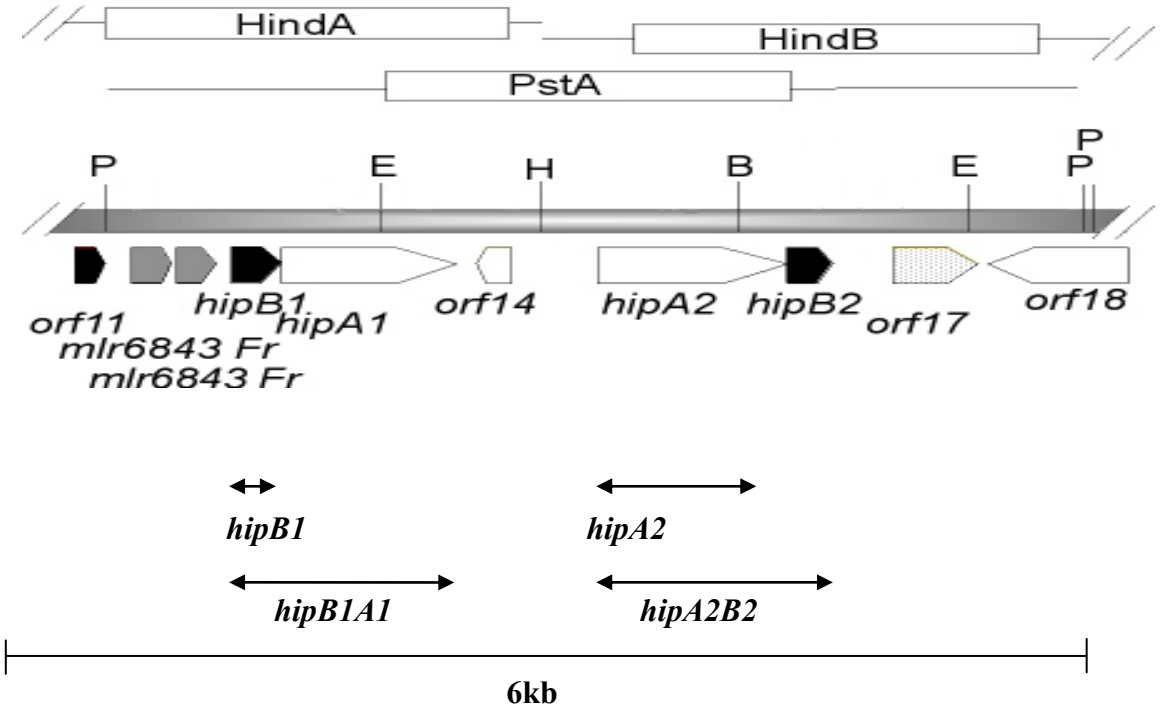


Figure 3.9: Diagrammatical representation of the HindA, HindB and PstA restriction fragments in pJW5 that had previously been shown to provide complementation to non-symbiotic strains, allowing them to form Dct+ mutants after prolonged incubation (Julie Weaver, personal communication). The HindA fragment contains the *hipB1A1* genes, HindB fragment contains the *hipA2B2* genes while the PstA fragment encompasses both *hipA1B1* and *hipA2B2* genes. Horizontal double arrows represent the gene fragments amplified by PCR, namely, *hipB1*, *hipA2*, *hipA1B1* and *hipA2B2*.

Table 3.2: Total number of colonies and revertants formed by R7ANS containing specified plasmids at dilutions ranging from 10^{-5} to 10^{-7} on SRDM.

Total number of colonies on SRDM				Number of revertants on SRDM		
Plasmid in R7ANS	Dilution/10^			Dilution/10^		
	-5	-6	-7	-5	-6	-7
pFAJ1700	349	135	23	0	0	1
pJW5	320	166	43	212	110	22
pUShipB1A1	327	135	23	0	0	0
pUShipB1	329	176	33	0	0	0
pUShipA2B2	329	134	23	0	0	1
pUShipA2	322	132	45	0	0	0
pUSHindA	268	122	34	0	0	0
pUSHindB	279	114	25	0	0	0
pUSPstA	322	133	26	0	0	2

Table 3.3: Total number of colonies and revertants formed by CJ3 containing specified plasmids at dilutions ranging from 10^{-5} to 10^{-7} on SRDM.

Total number of colonies on SRDM				Number of revertants on SRDM		
Plasmid in CJ3	Dilution/10^			Dilution/10^		
	-5	-6	-7	-5	-6	-7
pFAJ1700	312	156	29	0	0	0
pJW5	335	144	31	198	97	14
pUShipB1A1	240	110	19	0	0	0
pUShipB1	289	117	23	0	0	0
pUShipA2B2	277	111	22	0	0	0
pUShipA2	347	144	67	0	0	0
pUSHindA	320	116	27	0	0	0
pUSHindB	276	109	21	0	0	0
pUSPstA	213	98	18	0	0	0

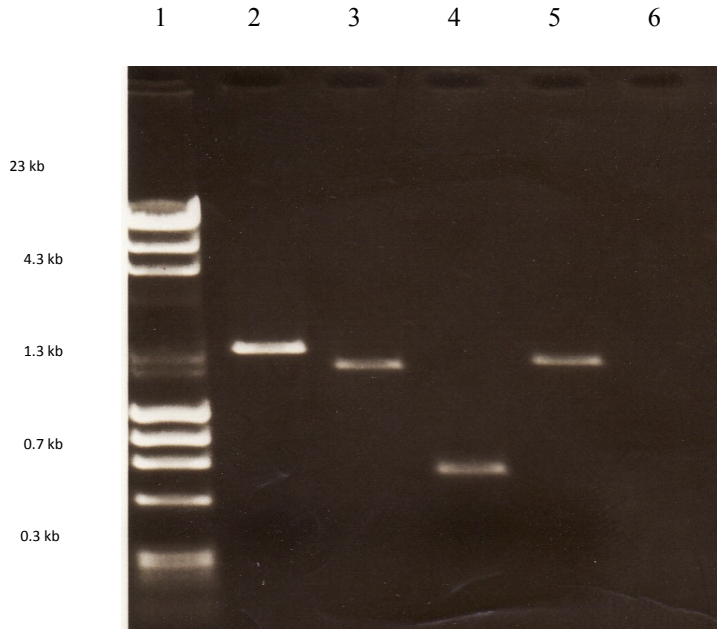


Figure 3.10: Agarose gel showing PCR products amplified from pJW5. Lane 1, λHindIII plus ΦX174 DNA Hae III marker. Lane 2, *hipA2B2* PCR product. Lane 3, *hipA2* PCR product. Lane 4, *hipB1* PCR product. Lane 5, *hipB1A1* PCR product. Lane 6, Negative control devoid of any DNA.

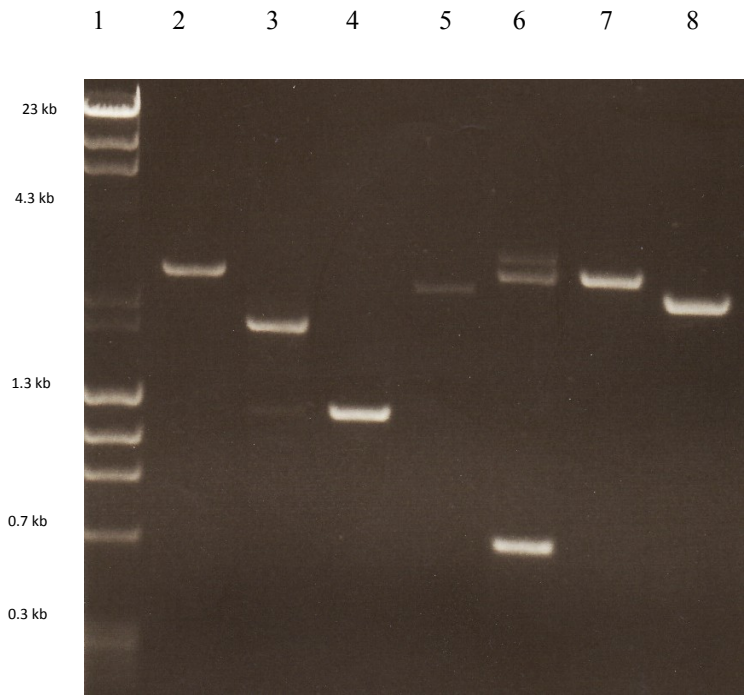


Figure 3.11: Agarose gel of PCR products from alkaline-lysis plasmid preps of pFAJ1700 harbouring the cloned *hipAB* genes. Lane 1, λHindIII plus ΦX174 DNA Hae III marker. Lanes 2 and 7, *hipA2B2* PCR products. Lane 4, *hipB1* PCR product. Lane 5, *hipB1A1* PCR product. Lane 8, *hipA2* PCR product. These products were subjected to sequencing to confirm clones. Products from Lanes 3 and 6 discarded due to multiple banding.

Two weeks after plating, the positive control plates (CJ3/pJW5, R7ANS/pJW5) showed colonies metabolising succinate while the negative controls and test plates only had microcolonies on the surface. At four weeks post plating, there was no phenotypic change on the negative control plates (except a few dct^+ R7ANS colonies appearing at the highest dilution (10^{-7})) and test plates, indicating that succinate was not being utilised and there had been no reversion event from $dct^- \rightarrow^+$. The experiment was repeated twice with similar results.

These results seemed strange, as the initial complementation experiments indicated that a single *hipAB* locus (contained within a larger restriction fragment cloned in pFAJ1700) was sufficient for mutation to succinate metabolism (Julie Weaver, personal communication). It was possible that the inserts had undergone point mutations after electroporation into the rhizobial strains. Hence plasmids were recovered from each of the CJ3 and R7ANS strains

and their inserts were amplified by PCR and sequenced. The results indicated that no such event had occurred.

The pJW5 plasmid gives a $Dct^- \rightarrow^+$ phenotype in both R7ANS and CJ3 but the above results suggest that *hipAB* operons were not responsible for the phenotype. It could be possible that there are other genes in addition to *hipAB* that may be involved in promoting the reversion to Dct^+. To test this hypothesis, it was decided to subclone appropriate restriction fragments of pJW5. This approach would allow for the 'zeroing' in on fragments that promote a Dct^+ phenotype.

3.2.1 Digest of pJW5

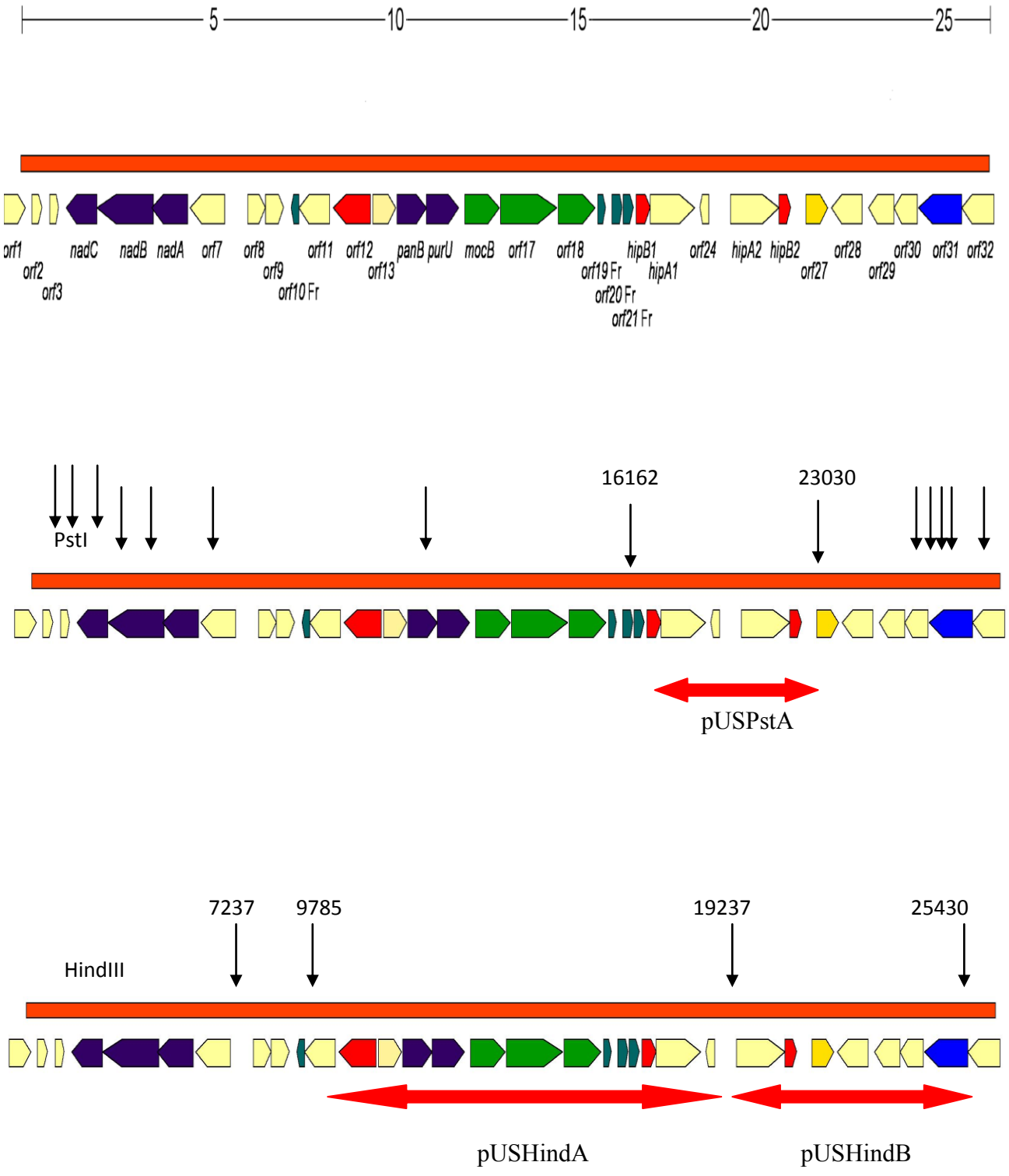
Vector NTI was used to create restriction profiles of pJW5 using common enzymes. The selection criteria for ideal enzymes included the following: the enzyme did not have multiple sites in the cloning vector; the enzyme digest gave regions encompassing the *hipAB* genes; and the enzyme digest gave regions that were outside the *hipAB* gene region.

Using these criteria as a guideline, BglII was chosen for a single digest and NheI/SacI for a single and double digest. A map of the regions is given below (**Figure 3.12**), along with PstI and HindIII digests.

The BglII-digested products were introduced into the cloning vector pProbe (Kan^R) while the NheI/SacI products were self-ligated in a pLAFR1 vector background. These products were then electroporated into *E. coli* S17 cells to confirm the clones. Once confirmed by restriction digest, plasmids were electroporated into rhizobial cells and the cells plated onto GRDM with the appropriate antibiotic. Once the cells had grown, they were then plated onto succinate media along with appropriate controls.

Three weeks post plating at 28°C, the positive control plates inoculated with CJ3/pJW5 and R7ANS/pJW5 showed mutants appearing on the surface of the agar (**Figure 3.13**). At this time none of the test plates which include R7ANS and CJ3 containing the digested products of pJW5, showed any signs of mutation (**Figure 3.13**). Only microcolonies were visible. These results consolidate previous findings that suggest no involvement of the *hipAB* operons in the mutator phenotype on pJW5. This is based on the fact that the BglII, NheI and SacI digests contain both the *hipA1B1* and *hipA2B2* genes and generous upstream and downstream

sequence (**Figure 3.12**). There must be other factors, not including *hipA* or *hipB*, on pJW5 that promote the mutator phenotype.



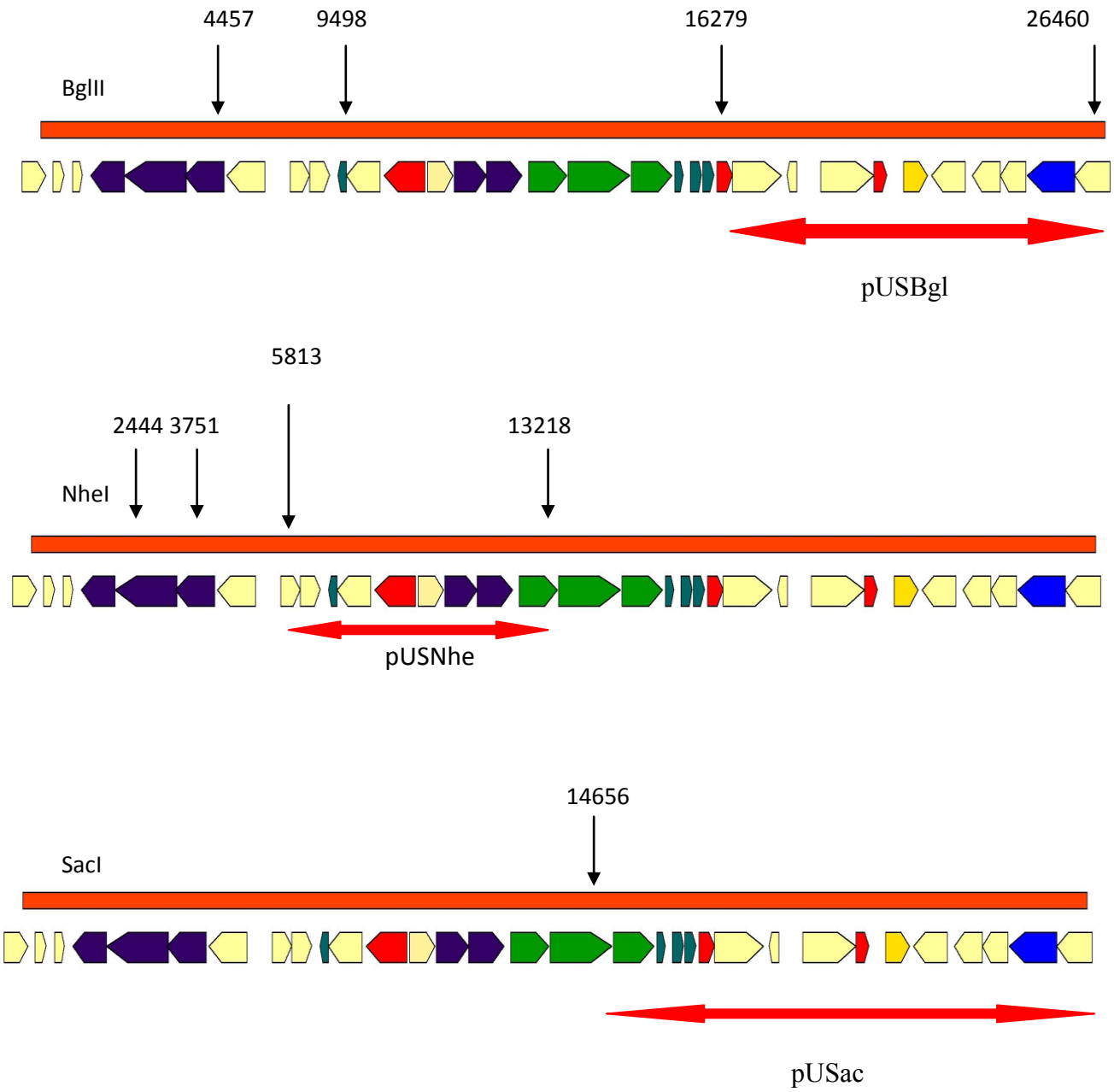


Figure 3.12: Restriction map of pJW5. Vertical arrows represent restriction sites and the numbers above them denote the position of the site on the plasmid. Red double arrows below each restriction map illustrate the regions of pJW5 that have been sub cloned and tested. The names of these plasmids are given below the arrows.

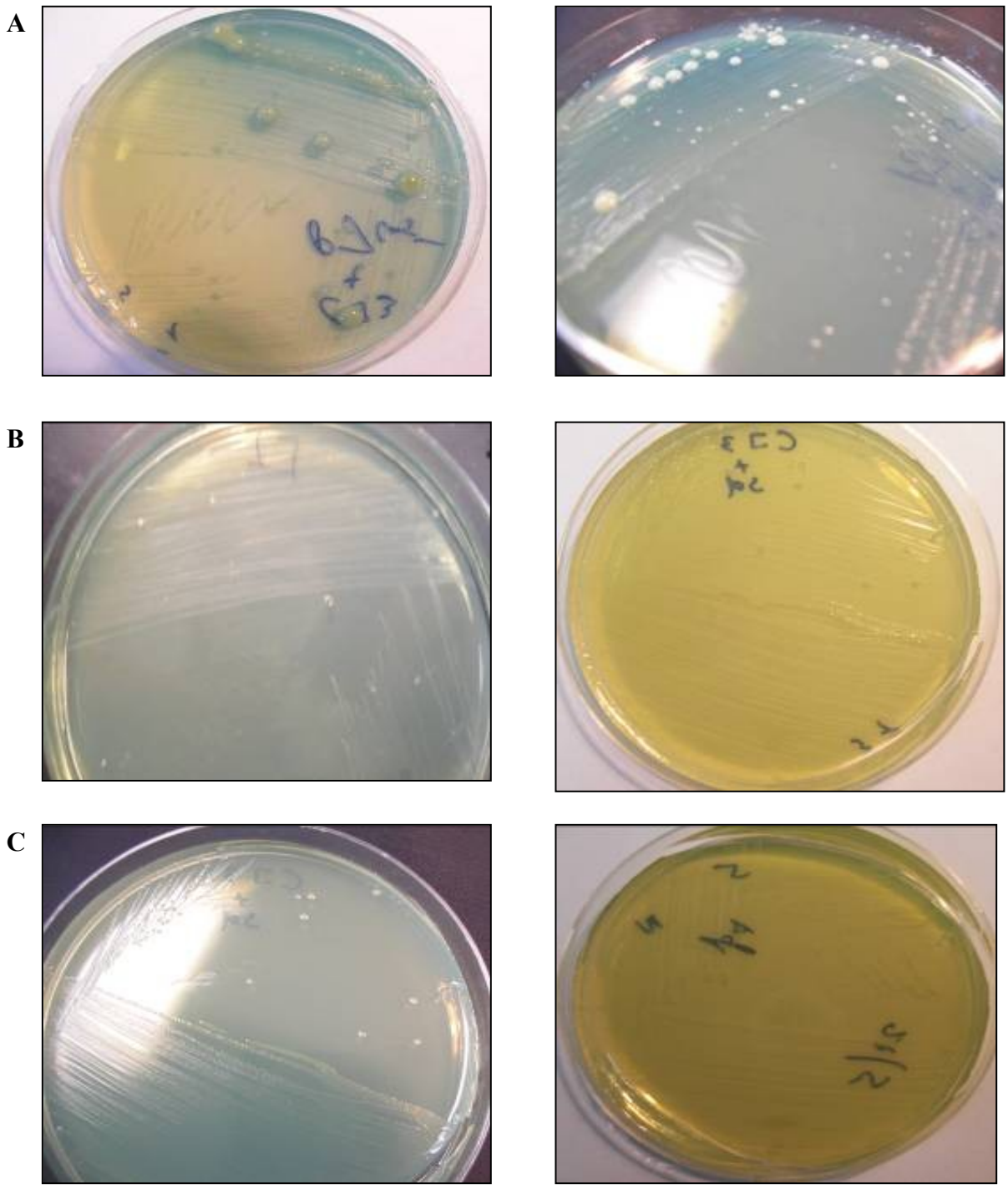


Figure 3.13: Photographs show **A** positive controls CJ3/pJW5 (left) , R7ANS/pJW5 (right); and test plates **B** R7ANS/pUSBgl (left) , CJ3/pUSNhe (right); and **C** CJ3/pUSac (left), R7ANS/pUSdd (right). All strains were grown on SRDM plates and photographed 3 weeks after plating. Positive controls showed mucoid Dct^+ colonies while no test plate showed any colonies utilizing succinate.

3.3 Knocking out *mfd*

Occurrence of mutations in the presence of succinate suggested that transcription may be involved in promoting a Dct^+ phenotype. As the host genes involved in mutational mechanism were unknown, I decided to focus on the *mfd* gene as it has been implicated in transcription-coupled repair (Ross *et al*. 2006). The availability of a completely sequenced genome from *M. loti* strain MAFF303099 (Kaneko *et al*. 2000) and the high degree of sequence similarity of this strain with *M. loti* strain R7A, prompted us to look for an *mfd* homologue in the MAFF303099 genome database. We located a strong homologue designated mll0827 (**Figure 3.14**) and designed primers flanking 1 kb regions on either side of the gene and an internal portion of the gene to facilitate knocking out the gene in R7ANS. The gene was deleted in the R7ANS strain by markerless deletion (**Figure 3.15**).

To test for the generation of a genuine *mfd* mutant, PCR was employed on four isolates subjected to in-frame deletion. Initial PCR was done with primer pairs 25mer5primeTCRF and 25mer3primeTCRF (**Table 2.3**) in order to amplify an internal portion of the *mfd* gene. Genuine mutants were identified as those that did not give a product with these primers as they are deleted of the internal portion of the gene (**Figure 3.16**). A second round of PCR was done on the candidate mutant strains with primer pairs mfdFORW250 and mfdREV250 (**Table 2.3**) which flank the *mfd* gene. Genuine mutants were identified as having a 500 bp size band after PCR as this indicates absence of any part the *mfd* gene and only flanking gene products (**Figure 3.17**). If the *mfd* gene was still present, no PCR product would be expected as the primers would be 4 kb apart.

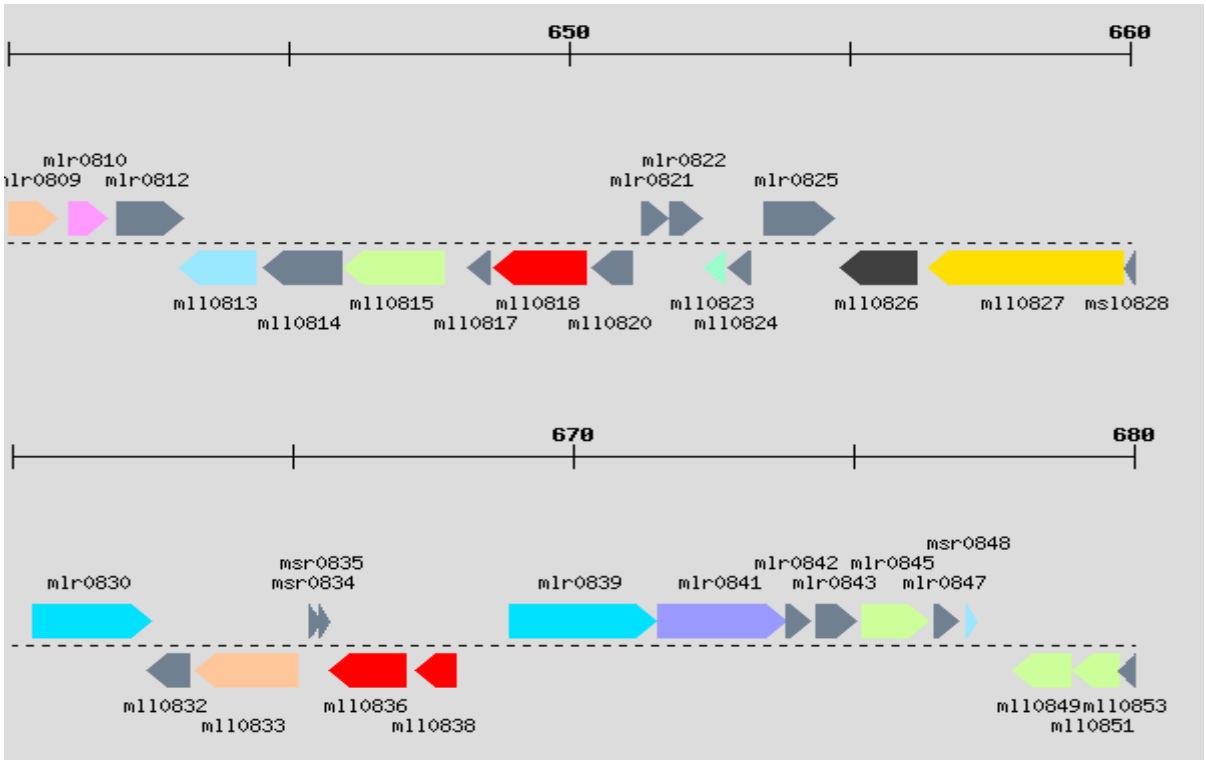


Figure 3.14: mll0827 is a hypothetical 3.5 kb Transcription Repair Coupling Factor TRCF protein-coding gene which is located downstream of a probable 2-dehydro-3-deoxyphosphoheptonate aldolase (msl0828) and upstream of an ATP-dependant DNA helicase (mll0826). Its discovery as a probable TRCF was made via a modified high-throughput yeast two-hybrid system which was aimed at studying protein-protein interactions in *M. loti* (Shimoda *et al*. 2008). It is a member of the *mfd* COG 1197, shows similarity over the full length protein and has an e value of < 1 x e-180. The gene encodes a 1165 amino-acid protein which shares homology to hypothetical TRCF proteins in other rhizobial strains such as *Bradyrhizobium japonicum* USDA 110 and *Sinorhizobium meliloti* 1021.

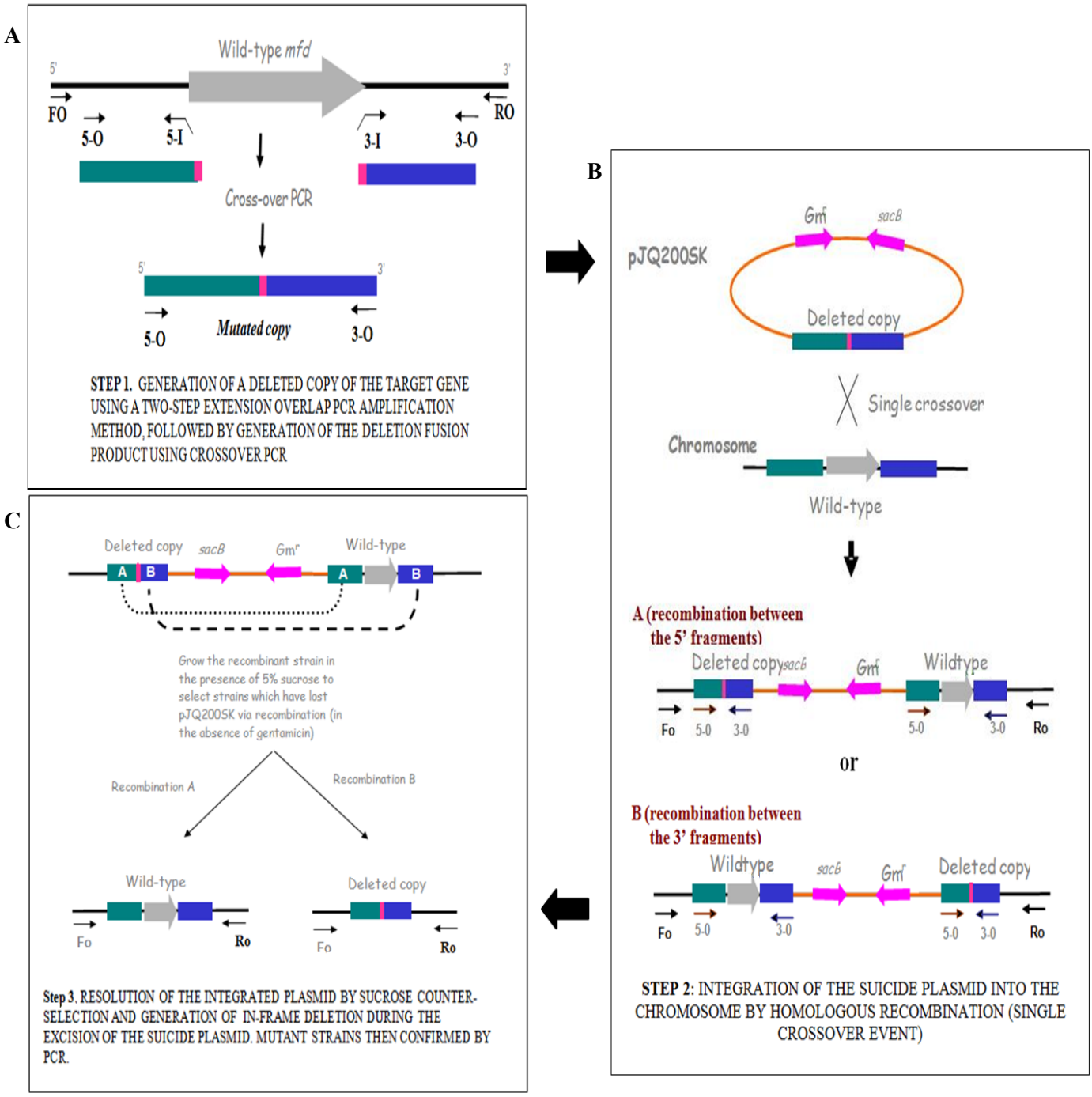


Figure 3.15: Diagrammatic representation of the strategy used to create markerless-deletion mutant strains. **A**. Deletion of the *mfd* gene was made by using overlap extension PCR with primers R7AmfdOELR and R7AmfdOERL (**Table 2.3**) followed by crossover PCR for fusion of the deleted product. **B.** A single crossover event via homologous recombination allowed integration of pJQ200SK into the chromosome of R7ANS. **C.** A second crossover event to remove pJQ200SK and the wild-type copy of the target gene was forced by plating single crossover clones on RDM containing 5% sucrose. Mutant strains were then confirmed by PCR (**Figure 3.16** and **Figure 3.17**).

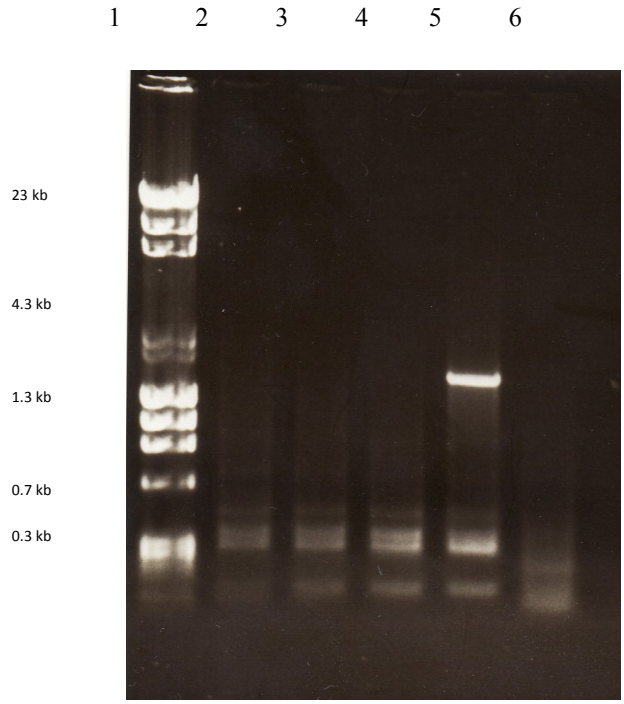


Figure 3.16: Agarose gel showing confirmation of mutants following PCR using primers 25mer5primeTCRF and 25mer3primeTCRF (**Table 2.3)** to amplify an internal portion of the *mfd* gene. Lane 1, λHindIII plus ΦX174 DNA Hae III marker. Lanes 2, 3, 4 and 6 represent genuine mutants that do not have a product with primers amplifying an internal portion of the gene. Lane 5 shows the positive control- strain R7ANS, which shows the presence of this portion of the *mfd* gene.

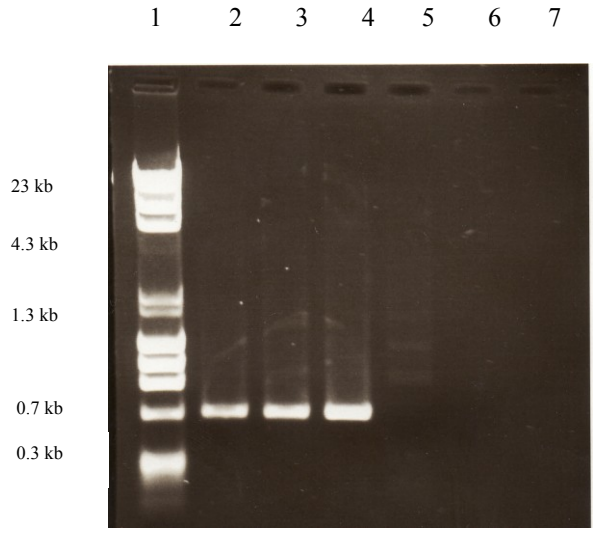


Figure 3.17: Agarose gel showing confirmation of mutants following PCR using primers mfdFORW250 and mfdREV250 (**Table 2.3**) to amplify regions flanking the *mfd* gene. Lane 1, λHindIII plus ΦX174 DNA Hae III marker. Lanes 2, 3 and 4 show genuine mutants that have a ~500 bp product from primers that amplify flanking portions of the *mfd* gene. Lane 5 shows a non-genuine mutant as there is no product following PCR.

3.3.1 The R7ANS *mfd* mutant shows an altered phenotype

pJW5 was introduced into the R7ANS *mfd* mutant via electroporation and following subsequent colony purification, was streak-plated onto succinate medium alongside the R7ANS/pJW5 positive control. Two weeks post incubation, Dct^+ colonies had arisen on the positive control. The *mfd* mutants, however, did not produce any Dct^+ colonies (**Figure 3.18**). Seventeen days post plating, however, the R7ANS *mfd* mutant with pJW5, started to show signs of Dct+ colonies but at much lower frequency than the positive control (**Figure 3.19**). This observation suggested that the loss of *mfd* may contribute to a reduction in numbers of mutant colonies. In order to test this hypothesis, dilution series of R7ANSΔ*mfd* and R7ANS/pJW5 were plated. Dilutions ranging from 10^{-5} to 10^{-7} were spread plated to determine the total number of colonies and number of revertants two weeks and three weeks post plating at 28°C. Results are described in **Table 3.4**, **Table 3.5** and **Figure 3.20**.

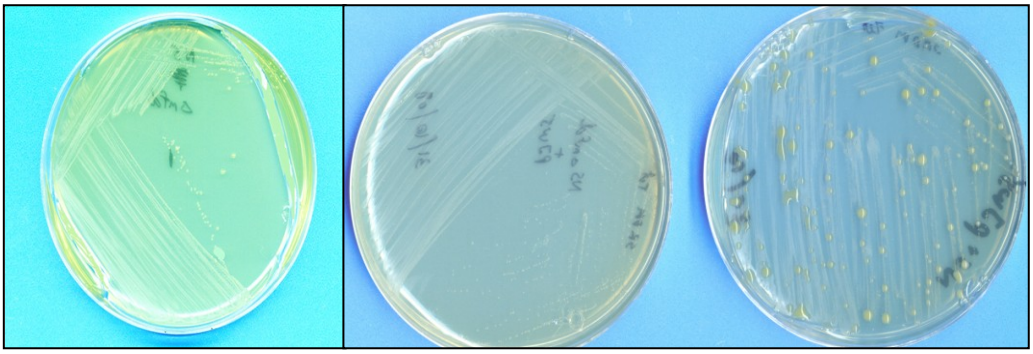


Figure 3.18: Plates of R7ANSΔ*mfd* (left), R7ANSΔ*mfd* /pJW5 (centre) and R7ANS /pJW5 (right), two weeks post plating. The positive control shows several Dct+ mutant colonies whereas the *mfd* mutant only formed microcolonies on the surface.

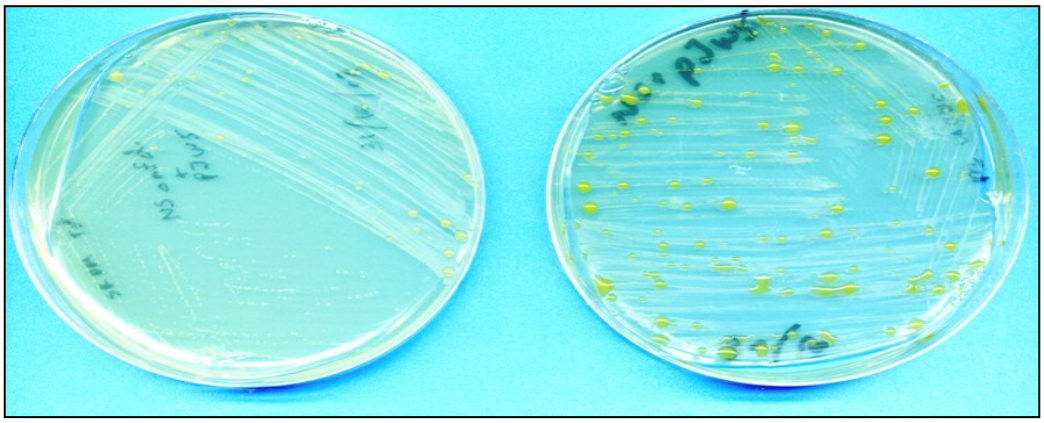


Figure 3.19: Plates of R7ANS Δ *mfd*/ pJW5 (left) and R7ANS / pJW5 (right), 17 days post plating. Mucoid Dct^+ colonies of the mutant strain are starting to appear on the surface of the medium.

Table 3.4: Total number of cells and revertant colonies seen on strain R7ANS/pJW5 at corresponding dilutions 2 and 3 weeks post plating.

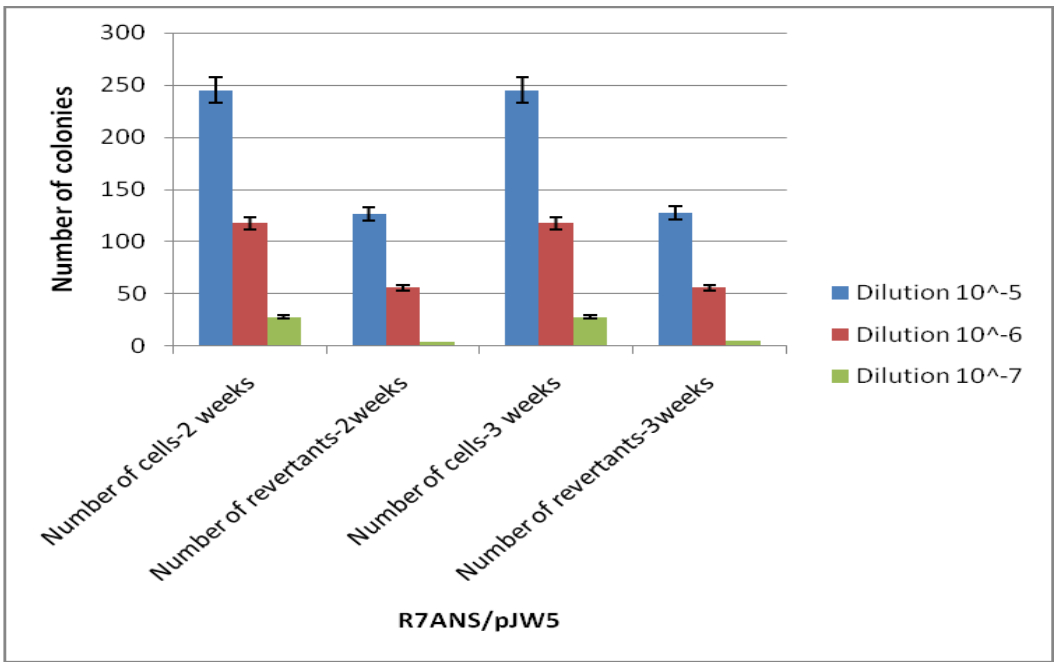
	R7ANS/pJW5			
Dilution/10^	Number of cells-2 weeks	Number of revertants-2 weeks	Number of cells-3 weeks	Number of revertants-3weeks
-5	245	127	245	128
-6	118	56	118	56
-7	28	4	28	5

Table 3.5: Total number of cells and revertant colonies seen on strain R7ANSΔ*mfd*/pJW5 at corresponding dilutions 2 and 3 weeks post plating.

R7ANS Δ *mfd* / pJW5				
Dilution/10^	Number of cells-2 weeks	Number of revertants-2weeks	Number of cells-3 weeks	Number of revertants-3weeks
-5	151	34	151	50
-6	43	3	43	8
-7	5	0	5	0

The dilutions listed in **Table 3.4** and **Table 3.5** were performed in triplicate and the average number of colonies is presented in the data. In the case of R7ANS/pJW5, it is observed that the number of cells and revertants two and three weeks after plating was almost identical. This was not the case for R7ANSΔ*mfd*/pJW5 where the number of revertants was greater 3 weeks post plating as compared to the numbers 2 weeks after plating. This result suggests that loss of *mfd* may decrease the frequency of mutation and hence delay the onset of revertants. A graphical representation of this data is given in **Figure 3.20.**

A



B

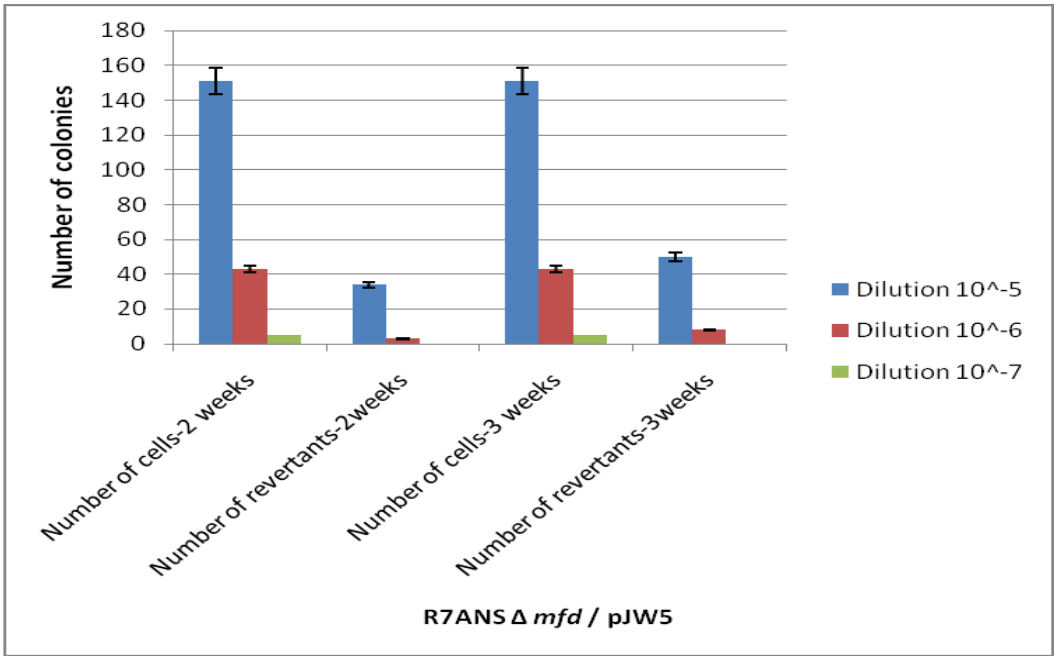


Figure 3.20: Bar graphs represent the number of colonies and revertants seen on SRDM two and three weeks after plating on strain (a) R7ANS/pJW5 and (b) R7ANSΔ*mfd*/pJW5 at varying dilutions. A significantly greater number of revertants are seen for strain R7ANSΔ*mfd*/pJW5 at 3 weeks as compared to 2 weeks post plating suggesting that the mutant strain has a decreased reversion rate from *dct*$^{-}$ to *dct*$^{+}$ as compared to the positive control strain, R7ANS/pJW5. Error bars are also shown on the graphs. Note that some error bars are so small that they cannot be seen on the graphs.

4 Discussion

4.1 The role of the *hipAB* operons in *Mesorhizobium loti*

Initial experiments in our laboratory identified a Tn5 insertion in the *hipB2* ORF on pJW5 which delayed the onset and reduced the frequency of Dct^+ colonies on SRDM (Weaver 2003). This gene was part of a *hipAB* operon which resembled a toxin-antitoxin (TA) module, identified in diverse bacterial species and proposed to be responsible for allowing cells to overcome stress by placing them in a dormant state (Lewis 2005). Two such *hipAB* operons were identified in *M. loti*, namely, *hipA1B1* and *hipA2B2*. Studies using restriction fragments harbouring each of these operons or both of them subcloned from pJW5 revealed that a single *hip* locus was sufficient to promote a Dct^- to Dct^+ phenotype. The mutator phenotype observed when pJW5 was introduced to non-symbiotic strains was hence attributed to the action of the *hipAB* genes placing cells in a persister state whereby they were able to compensate for metabolite insufficiency by undergoing adaptive mutation to produce revertants that could overcome selective stress (Weaver 2003; Weaver, personal communication). In this study I show that this proposed role of the *hipAB* genes is incorrect. The *hipAB* genes do not contribute to the mutator phenotype observed when pJW5 is introduced to non-symbiotic strains. The following paragraphs justify my research.

4.1.1 Overexpressing the *hipA* gene over its cognate antitoxin *hipB* has no effect on cell growth or viability

The *hipA1* and *hipA2* genes from CJ1 were cloned into an inducible expression vector pTH1227. By varying the amount of IPTG added, I was able to regulate the level of expression of these genes driven by the Ptac promoter (**Figure 3.3**). When introduced into non-symbiotic strains, however, the *hipA* genes did not demonstrate the effects that were observed with the positive control pJW5. This held true for both minimum and maximum expression of the genes from the inducible promoter. Interestingly, the vector pTH1227 and a few of the *hipA* plates showed evidence of some reversion predominately on high dilution plates (10^{-7}). Cells that are growing in higher dilutions are in conditions of greater stress as

there is an increased competition for available nutrients. One would hence expect to observe a greater number of revertant colonies on plates with a higher dilution due do the increased number of bacterial cells under stress. This was not the case in the present experiment. It could also be possible that the vector pTH1227 may be harbouring genetic loci that resemble a TA module. Many studies have shown that TA modules are essentially 'addiction molecules' that enable the cell to undergo programmed cell death when encountered with periods of starvation (Aizenman *et al*. 1996). Many of these addiction molecules have been identified in plasmids and are involved in killing cells that have lost the plasmid (Dao Thi *et al*. 2002, Lehnherr *et al*. 1993, Deane *et al*. 2004). A good example is the *ccd* addiction system that plays a crucial role in the stable maintenance of the *E. coli* F plasmid (Dao Thi *et al*. 2002). It codes for a stable toxin (CcdB) and a less stable antitoxin (CcdA). Both are expressed at low levels during normal cell growth. Upon plasmid loss, CcdB outlives CcdA and kills the cell by poisoning gyrase, an enzyme that unwinds double stranded DNA. In a similar fashion pTH1227 may harbour such TA modules that allow the cell to overcome selective stress by placing it in a dormant state. Essentially, the revertant colonies identified on the *hipA* plates (**Figure 3.6**) are not due to the effects of the *hipA* gene but probably the actions of pTH1227. It is also possible that these revertants represent the low background reversion frequency that strain R7ANS exhibits, however revertants on the R7ANS negative control plates were not observed (**Table 3.1**).

The overexpression study that I conducted also raised questions as to whether the *hipA* gene product represented a genuine toxin molecule as part of a TA module. The *hipAB* operons on pJW5 are similar (11.5% amino-acid identity) to the *E. coli hipAB* genes (Weaver 2003). Studies have shown that when the *E. coli hipA* gene is ectopically overexpressed cells become dormant and form persisters, in that they are viable but not culturable (Correia *et al*. 2006)(Korch and Hill 2006). During this persister state, macromolecular synthesis ceases and this is reversible by addition of the cognate antitoxin HipB (Korch and Hill 2006). In this study, however, cells that harboured overexpressed HipA molecules were still able to grow on SRDM agar and in GRDM and TY broths as shown in the GUS activity assay (**Figure 3.3**). Even at the highest concentration of IPTG, the cells were able to grow in these broths (as shown by increase in optical density in this assay) and hydrolysed PNPG. This observation could be due to the fact that the *hipA* genes were not expressed from their native promoter region. Furthermore, the occurrence of persister cells has not been reported for

M. loti. An interesting direction for future research would be to ascertain whether the *hipAB* genes on pJW5 represent a genuine TA module. This could be achieved by cloning the *hipA* genes, from pJW5 into *E. coli* and overexpressing them. Studies on cell growth, viability and susceptibility to antibiotics would ascertain the function and nature of the *hipA* gene in pJW5. Ability to rescue the cell by induction of *hipB* from pJW5 would also consolidate the knowledge about this module in *M. loti*.

4.1.2 The *hipAB* operons do not show any effects on mutation to Dct+ in non-symbiotic strains

The overexpression studies involved only the *hipA* genes being expressed under the control an inducible promoter regions. Taking this into consideration, the next part of my research focused on determining the effects of cloning the *hipA*, *hipB* and *hipAB* genes in non-symbiotic strains under the control of their native promoter regions. I also tested subclones of pJW5 that were claimed to provide a mutator phenotype in non-symbiotic strains. These clones contained one *hipAB* operon or both as restriction fragments. Once again, I observed that the *hipA*, *hipB* and *hipAB* genes, including the subclones, did not allow for mutation to Dct^+. It was observed that strains harbouring pJW5, however, had more than half the total cell population mutating to Dct^+ at all dilutions tested (**Table 3.2** and **Table 3.3**). These data consolidated the hypothesis that the *hipAB* genes are not involved in the Dct^- to Dct^+ phenotype observed when pJW5 is introduced into non-symbiotic strains.

To narrow down the regions of pJW5 that promote a mutator phenotype, further restriction fragments of pJW5 were subcloned (**Figure 3.12**). The BglII fragment (about 10 kb in size compared to pJW5 which is 26 kb in length) harboured both *hipAB* operons and about 1 kb downstream and 5 kb upstream of the genes. The SacI digest cut approximately 13 kb of pJW5, almost half of it, and this included about 3.5 kb upstream of the start of the *hipA1B1* gene. The NheI digest makes cuts further upstream of the SacI restriction site allowing for more sequence upstream of the *hipAB* operons. When these fragments were subcloned into non-symbiotic strains, it was observed that the cells were not able to mutate to succinate utilization. Since all DNA of pJW5 except for the left-most 5 kb has been subcloned, results indicate that the gene(s) responsible for the mutator phenotype on pJW5 are most probably located roughly 5 kb from the start of the plasmid (see **Figure 3.12**). This result also serves as another point of evidence against the *hipAB* genes promoting a mutator phenotype and

activating the cryptic *dctABD* genes. Further study should focus on characterising the first 4-6 kb segment of pJW5. This segment contains the *nadABC* genes which are required for the synthesis of the nicotinamide adenine dinucleotide (NAD) intermediate nicotinamide mononucleotide (John Sullivan, personal communication). Since my experimental work was completed, others in the lab have shown that the *nadABC* genes are responsible for the mutator phenotype (John Sullivan, personal communication).

4.2 Characterising an *mfd* mutant in R7ANS

Earlier studies in our laboratory established that mutation to Dct^+ in non-symbionts did not require *recA* or *relA* (John Sullivan, personal communication). Both these genes play central roles during periods of stress such as damage to DNA or nutrient insufficiency respectively (Magnusson *et al.* 2005) (Better and Helinski 1983). In a search for genes that could play a role in the mutator phenotype observed in *M. loti*, I came across the *mfd* gene. This gene was of particular interest for this study as it has been implicated to be involved in repair of DNA and adaptive mutagenesis amongst others (Ross *et al.* 2006). Ross and co-workers demonstrated that an auxotrophic strain of *B. subtilis* devoid of the *mfd* gene exhibited a lower reversion to prototrophy than the wild-type strain during periods of starvation (Ross *et al.* 2006). This result was puzzling as *mfd* is involved in transcription coupled repair of DNA and strains deleted of this gene should rightfully show more adaptive mutants under stress, due to the increase in mutation rates. In this study, I observed a similar occurrence.

An *mfd* mutant of R7ANS was constructed and plated on SRDM alongside R7ANS/pJW5 (positive control) and R7ANSΔ*mfd*/pJW5. Although the *mfd* mutant did not exhibit any mutation to Dct^+, the R7ANSΔ*mfd*/pJW5 colonies exhibited different growth behaviour compared to the positive control (see **Figure 3.18, Figure 3.19** and **Figure 3.20**). Generally, the Dct^+ mutant colonies took longer to appear on plates of the *mfd* mutant strain as compared to the strain with *mfd* intact. R7ANS/pJW5 showed revertants two weeks post plating, while the *mfd* mutant took seventeen days to start showing signs of reversion. **Table 3.4** and **Table 3.5** show the number of colonies and revertants on R7ANS/pJW5 and R7ANSΔ*mfd*/pJW5, two and three weeks post plating. Taking the number of revertants for both strains at the lowest dilution (10^{-5}) at three weeks post plating and dividing it by the total number of

colonies for each strain, gives an estimate of the ratio of revertants to colonies for each strain as follows:

For R7ANS/pJW5: Revertants/colonies = 128/245 = **0.522** (at three weeks post plating)

For R7ANSΔ*mfd*/pJW5: Revertants/colonies = 50/151 = **0.331** (at three weeks post plating)

It is also possible to estimate the change in the rate of reversion in both strains. This can be achieved by taking the number of revertants for both strains at the lowest dilution (10^{-5}) at two weeks post plating and dividing it by the total number of colonies for each strain and comparing this to the ratio obtained for three weeks post plating.

For R7ANS/pJW5: Revertants/colonies = 127/245 = **0.518** (at two weeks post plating)

For R7ANSΔ*mfd*/pJW5: Revertants/colonies = 34/151 = **0.225** (at two weeks post plating)

From this data, we can contrast the change in the rate of reversion in both strains at two weeks and three weeks post plating. For R7ANS/pJW5, we have a change in the rate of reversion from 0.518 at two weeks to 0.522 at three weeks which gives a percentage increase of approximately 0.8%. This is a very minute increase as compared to R7ANSΔ*mfd*/pJW5 which increases its rate of reversion by almost 32% over this period of time.

Clearly, the numbers suggest that after 3 weeks, half of the total colonies on the R7ANS/pJW5 plates give rise to Dct^+ mutants as compared to a third on R7ANSΔ*mfd*/pJW5. This observation indicates that a loss of *mfd* in R7ANS may cause a reduction in the *rate* of reversion to Dct^+ in addition to delaying their appearance and decreasing their numbers. Rate is a function of time, therefore a useful experiment would involve monitoring the daily numbers of revertants for the mutant strain and the positive control over a period of a few weeks. A good starting point for future work would also be to elucidate the effects of deleting the *mfd* gene on mutation to succinate utilisation in another non-symbiotic strain such as CJ3. In this way, we can be quite certain that loss of *mfd* plays a direct role in the mutation event and that observations in this study are not merely interactions of *mfd* with a particular strain of bacteria.

It is also of great interest to elucidate the reason as to why there should be a delay and decrease in numbers of revertant colonies with the *mfd* mutant. As mentioned earlier, loss of *mfd* would theoretically result in an increase in overall mutation rates and thus aid in adaptive

mutagenesis to a mutator phenotype. Ross and colleagues believe that their observation of a reduction in revertants in the three alleles tested (**Figure 4.1**) in the *mfd*-deficient strain can be explained by approaching the situation at a transcriptional level.

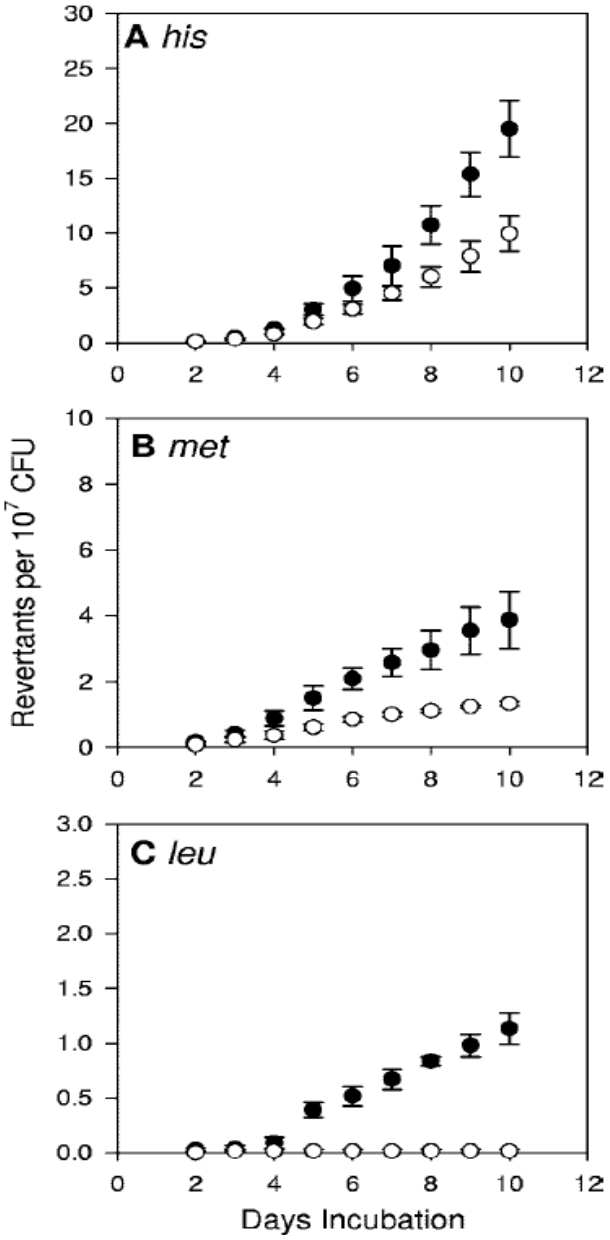


Figure 4.1: Stationary-phase reversion frequency. Number of revertants to histidine (A), methionine (B), or leucine (C) prototrophy occurring during prolonged incubation at 37°C on minimal selective media is shown for normal *B. subtilis* strain (closed circles) or the *mfd* deficient strain (open circles). Error bars represent 1 standard error from three independent trials. Figure excerpted from (Ross *et al*. 2006).

This inference stems from the basic knowledge that Mfd connects the processes of DNA repair and transcription such that transcriptional derepression could be associated with the ability to accumulate mutations. Studies have shown that the process of transcription leaves coding DNA unprotected and prone to form secondary structures and these features result in introduction of permanent DNA changes by subsequent replication (Wright 2000). Derepression of the three alleles shown in **Figure 4.1** could thus have lead to the observation of the occurrence of revertants in these alleles. The differences in the number of mutations that accumulated in the test alleles are possibly due to differences in the rates of transcription and the susceptibility to deamination and/or the formation of secondary structures (Ross *et al.* 2006). It is not clear at this point whether this inference holds true for the reversions seen with R7ANS. Instead of acting directly with the derepression of the *dctABD* genes, it is possible that the *mfd* gene interferes with the transcription of gene(s) responsible for the reversion phenotype on pJW5.

Another plausible explanation for the decrease in revertant numbers is transcriptional bypass. This process involves polymerase bypassing unrepaired DNA damage such as bulky lesions (Holmquist 2002). Studies have shown that the *mfd* gene facilitates transcriptional bypass (Saxowsky 2006), resulting in the formation of mutant mRNA. The altered mRNA would then give rise to a varied growth phenotype from a modified translated polypeptide. A loss of *mfd* would mean that DNA replication would ensue without proper repair mechanisms and a heritable change at the site of the lesion could result (Ross *et al.* 2006). Similarly, in the case for the R7ANSΔ*mfd*/pJW5 strain, a reduction in the number of revertants could be due to the accumulation of mutant peptides that cause a cessation to the effects of the gene(s) that enhance the mutator phenotype. The first step in testing this hypothesis would be to determine the levels of transcriptional bypass in the R7ANSΔ*mfd*/pJW5 strain. A reduction in the levels of bypass would confirm the role of *mfd* in mutagenesis and transcription in these strains.

In all, this thesis has provided a framework for the future research of mutagenesis in *M. loti*. Studies on activation of the cryptic *dct* genes should focus largely on characterising the first 4-6 kb of pJW5 for gene(s) that promote the mutator phenotype. Although it is now clear that the *hipAB* genes play no role in the reversion event, an understanding of whether these genes constitute a TA module and their effects in *M. loti* would certainly progress our knowledge about bacterial evolution especially that of conserved genes between unrelated species. This

also hold true for the *mfd* gene, which, like *hipAB*, is found in a diverse number of bacterial species. A relationship between loss of *mfd* and the frequency of revertants, in a R7ANS/pJW5 background has been established in this work and future studies with other non-symbiotic strains such as CJ3 could yield interesting results.

Altschul, S.F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucliec Acids Res*. **25** : 3389-3402.

Andersson, D. I., E. S. Slechta, and J. R. Roth. 1998. Evidence that gene amplification underlies adaptive mutability of the bacterial *lac* operon. *Science*. **282**:1133–1135.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology*. **84**: 188-98.

Black, D.S.,Irwin ,B. and Moyed, H.S. 1994. Auto regulation of *hip*,an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. *Journal of Bacteriology*. **176**: 4081–4091.

Bodogai, M., Ferenczi, Sz., Bashtovyy, D., Miclea, P., Papp, P. and Dusha I. 2006. The *ntrPR* operon of *Sinorhizobium meliloti* is organized and functions as a Toxin-Antitoxin module. *Molecular Plant-Microbe Interactions*. **19**: 811-822.

Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology*. **41**: 459-72.

Bringhurst, R. M., and Gage, D. J. 2002. Control of Inducer Accumulation Plays a Key Role in Succinate-Mediated Catabolite Repression in *Sinorhizobium meliloti*. *Journal of Bacteriology*. **184** : 5385–5392.

Cairns, J., and Foster, P. L. 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. *Genetics*. **128**:1433-1440.

Cairns, J., Overbaugh, J. and Miller, S. 1988. The Origin of Mutants. *Nature*. **335**:142-145.

Cheng, J. , Sibley, C. D., Zaheer, R. and Finan, T.M. 2007. A *Sinorhizobium meliloti minE* mutant has an altered morphology and exhibits defects in legume symbiosis. *Microbiology*. **153**:375-387.

Choder, O. A., and Wright, A. 1993. Transcriptional Regulation of the *bgl* operon of *Escherichia coli* involves phosphotransferase system-mediated phosphorylation of a transcriptional antiterminator. *Journal of Cellular Biochemistry*. **51** :83-90.

Crick, F. 1970. Central dogma of molecular biology. Nature. **227**: 561-563.

Darwin, C. 1872.On the Origin of Species by Means of Natural Selection. *John Murray*, London. 6th ed.

Deaconescu, A, M., Chambers, A. l., Smith, A. J., Nickels, B. E., Hochschild, A., Savery, N. J. and Darst, S. A. 2006. Structural basis for bacterial Transcription-Coupled DNA Repair. *Cell*. **124**: 507-520.

Deaconescu, A., M., Savery, N. and Darst, S., A. 2007. The bacterial transcription repair coupling factor. *Current Opinion in Structural Biology* . **17**: 96-102.

Dombrecht, B., Vanderleyden, J., and Michiels J. 2001. Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. *Molecular Plant-Microbe Interactions*. **14**:426-430.

Dusha, I., Bakos, Á., Kondorosi, Á., de Bruijn, F. J., and Schell, J. 1989 . The *Rhizobium meliloti* early nodulation genes (*nodABC*) are nitrogen regulated: Isolation of a mutant strain with efficient nodulation capacityon alfalfa in the presence of ammonium . *Molecular and General Genetics*. **219:**89-96.

Falla, T. J., and Chopra, I. 1998. Joint tolerance to β-lactam and fluoroquinolone antibiotics in *Escherichia coli* results from overexpression of *hipA*. *Antimicrobial Agents and Chemotherapy*. **42**:3282-3284.

Feliciello, I., and Chinali, G. 1993. A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. *Analytical Biochemistry*. **212**: 394-401.

Foster, P. L. 2007. Stress-Induced Mutagenesis in Bacteria. *Critical Reviews in Biochemistry and Molecular Biology*. **42**:373–397.

Foster, P.L. 2004 . Adaptive mutation in *Eschercia coli. Journal of Bacteriology*. **186**:4846-4852.

Foster, P.L. 1997. Nonadaptive mutations occur on the F' episome during adaptive mutation conditions in *Escherichia Coli.* . *Journal of Bacteriology*. **179**:1550-1554.

Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel F. M. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene*. **18**: 289-296.

Frost, L. S., and Manchak J. 1998. F - phenocopies: characterization of expression of the F transfer region in stationary phase. *Microbiology* . **144**:2579-2587.

Gerdes, K., Christensen, S.K. and Olesen, A.L. 2005. Prokaryotic Toxin-Antitoxin stress response loci. *Nature Reviews Microbiology*. **3**:371-382.

Gonzalez-y-Merchand, J., A., Estrada-Garcia, I., Coleston, M. J. and Cox, R. A. 1996. A novel method for the isolation of mycobacterial DNA. *FEMS Microbiology Letters*. **135**: 71-77.

Hall, B.G. 1990. Spontaneous point mutations that occour more often when advantageous than when neutral. *Genetics*. **126**: 5-16.

Hall, B.G., S. Yokohama, and D.H. Calhoun. 1983. Role of cryptic genes in microbial evolution. *Molecular Biology and Evolution*. **1**: 109-124.

Hendrickson, H., Slechta E.S., Bergthorsson U., Andersson D.I., and Roth J.R. 2002. Amplification-mutagenesis: Evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. *Proceedings of the National Academy of Sciences*. **99**: 2164-2169

Holmes, D., S., and Quigley, M. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry*. **114**: 193-197.

Holmquist, G.P., and Maher, V.M. 2002. The bypass of DNA lesions by DNA and RNA polymerases. *Mutation Research*. **510**(1-2): 1-7

Jefferson R.A., Burgess S.M., and Hirsh D. 1986. β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proceedings of the National Academy of Sciences*. **83**:8447–8451.

Kahn, S. N., and Yurgel M.L. 2005. *Sinorhizobium meliloti dctA* mutants with partial ability to transport dicarboxylic acids. *Journal of Bacteriology*. **187**: 1161-1172.

Kaneko, T., Nakamura, Y., Sato, S. *et al.* 2000. Complete Genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Research*. **7**: 331-338.

Keren, I., Shah, D., Spoering, A., Kaldalu, N. and Lewis, K. 2004. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *Journal of Bacteriology*. **186**: 8172-8180.

Kivisaar, M. 2003. Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. *Environmental Microbiology*. **5**: 814-827.

Korch, S. B., and Hill, T.M. 2006. Ectopic overexpression of wild-type and mutant *hipA* genes in *Escherichia coli*: effects on macromolecular synthesis and persister formation. *Journal of Bacteriology*. **188**:3826-3836.

Korch, S. B., Henderson, T.A., and Hill T.M. 2003. Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Molecular Microbiology*. **186**:8172-8180.

Layton, J.C., and Foster P.L. 2003. Error prone DNA Pol IV is controlled by the stress response sigma factor , RpoS, in *Escherichia coli*. *Molecular Microbiology*. **50**:549-561.

Lewis, K. 2005. Persister cells and the riddle of biofilm survival. Biochemistry. **70**:327-336.

Lewis, K. 2007. Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology*. **5**:48-56.

Liu, Y. N., Tang, J. L., Clarke, B. R., Dow, J. M., and Daniels, M. J. 1990. A multipurpose broad host range cloning vector and its use to characterise an extracelluar protease gene of *Xanthomonas campestris pathovar campestris*. *Molecular and General Genetics*. **220**: 433-440.

Lou, C., Li .Z., and Ouyang, Q. 2008. A molecular model for persister in *Escherichia coli*. *Journal of Theoretical Biology*. **255** : 205–209.

Luria, S. E., and Delbruck, M. 1943. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics*. **28**:491–11.

Madan, R., Kolter, R., and Mahadevan, S. 2005. Mutations that activate the silent *bgl* operon of *Escherichia coli* confer a growth advantage in stationary phase. *Journal of Bacteriology*. **187**: 7912-7917.

Madan, R., Moorthy, S., and Mahadevan, S. 2008. Enhanced expression of the *bgl* operon of *Escherichia coli* in the stationary phase. *FEMS Microboilogy*. **288**: 131–139.

Magnusson, L. , U., Farewell A., and Nystrom, T. 2005. ppGpp: a global regulator in *Escherichia coli. TRENDS in Microbiology*. **13**: 236-242.

Miller, J.H. 1972. Experiments in molecular genetics. *Cold Spring Harbor Laboratory*, Cold Spring Harbor, New York.

Miller, V. L., and Mekalanos, J. J. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires *toxR* . *Journal of Bacteriology*. **170**: 2575-2583.

Miller, W. G., Leveau, J. H.J., and Lindow S. E. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Molecular Plant-Microbe Interactions*. **13**: 1243-1250.

Moyed, H. S., and Bertrand, K., P. 1983. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *Journal of Bacteriology*. **155**:768-775.

Oláh, B., Kiss, E., Györgypál, Z., Borzi, J., Cinege, G., Csanádi, G., Batut,J., Kondorosi, A. and Dusha, I. 2001. Mutation in the *ntrR* gene, a memberof the *vap* gene family, increases the symbiotic efficiency of *Sinorhizobium meliloti. Molecular Plant-Microbe Interaction*. **14**:887-894.

Oliver, J.D. 2005. The Viable but Nonculturable State in Bacteria . *Journal of Microbiology*. **43**:93-100 .

Quandt, J., and Hynes, M. F. 1993. Versatile suicide vectors allow direct selection for gene replacement in gram negative bacteria. *Gene*. **127**: 15-21.

Ramsay, J. P., Sullivan, J. T., Gabriella, S. S., Lamont, I. L., and Ronson, C. W. 2006. Excision and transfer of the *Mesorhizobium loti* R7A symbiosis island requires an integrase

IntS, a novel recombination directionality factor RdfS, and a putative relaxase RlxS. *Molecular Microbiology*. **62**: 723 - 734.

Roberts, J., and Park, J. S. 2004. Mfd, the bacterial transcription repair coupling factor:translocation, repair and termination. *Current Opinion in Microbiology*. **7**:120–125.

Robinson, J. B., and Bauer, W. D. 1993. Relationships between C4 dicarboxylic acid transport and chemotaxis in *Rhizobium meliloti*. *Journal of Bacteriology*. **175**: 2284-2291.

Ronson, C. W., Lyttleton, P., and Robertson, J. G. 1981. C4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proceedings of the National Academy of Sciences*. **78**: 4284-4288.

Ronson, C. W., Nixon, B. T., Albright, L. M. and Ausubel, F. M. 1987. *Rhizobium meliloti ntrA* (*rpoN*) gene is required for diverse metabolic functions. *Journal of Bacteriology*. **169**: 2424-2431.

Ronson, C.W., and Sullivan, J. T. 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proceedings of the National Academy of Sciences.* **95**: 5145-5149.

Rosche, W. A., and Foster P. L. 1999. The role of transient hypermutators in adaptive mutation in *Escherichia coli*. *Proceedings of the National Academy of Sciences.* **96**:6862-6867.

Rosenberg, S. M. 2001.Evolving Responsively: Adaptive Mutation. *Nature Reviews Genetics*. **2**:504-515.

Ross, C., Pybus, C.,Pedraza-Reyes, M., Sung, H., M., Yasbin, R. E., and Robleto, E. 2006. Novel role of *mfd*: Effects on stationary-phase mutagenesis in *Bacillus subtilis*. *Journal of Bacteriology*. **188**: 7512–7520.

Roth, J. R., Kugelberg, E., Reams, A.B., Kofoid, E. and Andersson, D., I. 2006.Origin of mutants under selection: The adaptive mutation controversy. *Annual Review of Microbiology*. **60**:477-501.

Sankar, T.S., Neelakanta,G., Sangal,V., Plum,G., Achtman M., and Schnetz K. 2009. Fate of the H-NS–repressed *bgl* operon in evolution of *Escherichia coli*. *Public Library of Science Genetics*. **5**: 1-11.

Savery, N. J. 2007. The molecular mechanism of transcription-coupled DNA repair. *TRENDS in microbiology*. **15**: 326-333.

Saxowsky, T.T., and Doetsch P.W. 2006. RNA polymerase encounters with DNA damage: Transcription-coupled repair or transcriptional mutagenesis. *Chemical Reviews*. **106**: 474-488.

Sheng, Y., Manchino, V. and Birren, B. 1995. Transformation of *Escherichia coli* with large DNA molecules by electroporation. *Nucliec Acids Res.* **23**: 1990-1996.

Shimoda, Y., Shinpo, S., Kohara, M., Nakamura, Y., Tabata, S., and Sato, S. 2008. A large scale analysis of protein-protein interactions in the nirogen fixing bacterium *Mesorhizobium-loti*. *DNA Research*. **15**:13-23.

Stumpf, J. D., Poteete , A.R., and Foster, P.L. 2007. Amplification of *lac* cannot account for adaptive mutation to Lac^+ in *Escherichia coli*. *Journal of Bacteriology*. **189**:2291-2299.

Sullivan, J. T., Patrick, H. N., P. van Berkum, and Ronson, C. W. 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. *Applied Environmental Microbiology*. **62**: 2818-2825.

Sullivan, J. T., Trzebiatowski, J. R., Cruickshank, R. W., Gouzy, J., Brown, S. D., Elliot, R. M., Fleetwood, D. J., McCallum, N. G., Rossbach U., Stuart, G. S., Weaver, J. E., Webby, R. J., F. J. de Brujin, and Ronson, C. W. 2002. Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A." *Journal of Bacteriology*. **184**: 3086-3095.

Sung, H. M., and Yasbin, R.E. 2002. Adaptive, or stationary-phase, mutagenesis, a component of bacterial differentiation in *Bacillus subtilis*. *Journal of Bacteriology*. **184**: 5641-5653.

Tompkins, J. D., Nelson J. E., Hazel J. C., Leugers S. L., Stumpf J. D., and Foster P. L. 2003. Error-prone polymerase, DNA polymerase IV, is responsible for transient hypermutation during adaptive mutation in *Escherichia coli*. *Journal of Bacteriology* . **185**: 3469-3472

Torkelson, J., Harris, R.S., Lombardo M.J. , Nagendran J., Thulin C., and Rosenberg S. M. 1997. Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation. *EMBO Journal.* **16**:3303-3311.

Weaver, J.W. 2003. Ph D Thesis. Cryptic *dct* genes of *Mesorhizobia.* Dunedin: *University of Otago*.

Wilson, K., J., Jefferson, R.A., and Hughs, S.G. 1992. The *Escherichia coli gus* operon induction and expression of the *gus* operon in *E. coli* and the occurrence and use of GUS in other bacteria. In GUS Protocols: Using the GUS gene as a Reporter of Gene Expression , by **Gallagher, S.R.** 7–22. London: *Academic Press*.

Witkin, E. M. 1966. Radiation-induced mutations and their repair. *Science*. **152**: 1345-1353.

Wrande, M., Roth, J. R., and Hughes, D. 2008. Accumulation of mutants in ‘‘aging’’ bacterial colonies is due to growth under selection, not stress-induced mutagenesis. *Proceedings of the National Academy of Sciences*. **105**: 11863–11868.

Wright, B. E. 2000. A biochemical mechanism for nonrandom mutations and evolution. *Journal of Bacteriology*. **182**:2993-3001

Yurgel, S.N. and Kahn, M.L. 2004. Dicarboxylate transport by *rhizobia*. *FEMS Microbiology Reviews*. **28**:489–501.

A

Growth media

Media were made up in distilled water and autoclaved at 121°C (15 psi) for 15 min on a liquid cycle. When solid culture media was required, GIBCO-BRL bacteriological agar was added to broth to give concentration of 1.5% before autoclaving.

Luria-Bertani (LB)

(Miller 1972)

Per 1 L, add

10 g Tryptone

5 g Yeast extract

5 g NaCl

Tryptone yeast (TY)

(Beringer 1974)

Per 1 L, add

5 g Tryptone

3 g Yeast extract

2 ml $CaCl_2.6H_2O$ (concentration 0.65 g/ml)

Rhizobium Defined Media (RDM)

(Ronson, *et al.* 1987)

To 900 ml distilled water, add

- 10 ml Salts (25 g $MgSO_4.7H_2O$, 2 g $CaCl_2.2H_2O$, 1.5 g FeEDTA, 20 g NaCl per liter)
- 10 ml BTB (concentration 2 mg/ml)
- 6 ml NH_4Cl (concentration 18g/L)
- 1 ml Trace elements (3 mg $ZnSO_4.7H_2O$, 40 mg $Na_2MoO_4.2H_2O$, 50 mg H_3BO_3, 40 mg $MnSO_4.4H_2O$, 4 mg $CuSO_4.5H_2O$, 1 ml $CoCl_2.6H_2O$ per 200 ml)
- 100 mg L-histidine

For GRDM, adjust pH to 6.5-7 with 2 M KOH and final volume 1L

For SRDM, add 50 mM MES (morpholineethanesulfonic acid) , adjust pH 6.1-6.3 using solid KOH and final volume to 1L

After the medium has been autoclaved, allow to cool and then add;

- 20 ml 20% Glucose (autoclave) for GRDM or 10mM Succinate (autoclave) for SRDM
- 10 ml Phosphates (20 g K_2HPO_4 and 20g KH_2PO_4 in 200ml distilled water, autoclave)
- 1 ml Vitamins if required (50 mg Thiamine HCl, 100 mg Ca Panthothenate and 1 ml Biotin [1 mg/ml], made up to 50 ml with sterile MiliQ water and filter sterilize)
- Antibiotics and antifungal agents if required

B

Buffers and Solutions

1 M Tris [pH8]

Add 121.1 g tris (hydroxylmethyl) aminomethane to 800 ml distilled water and add approximately 42 ml HCl to adjust to pH 8. Then, add appropriate amount of distilled water to give the final volume of 1 L. Autoclave

3 M Sodium acetate [pH 5.2]

Add 246.09 g sodium acetate (NaAc) anhydrous (molecular weight 82.03) to 800 ml of distilled water, adjust to pH 5.2 with glacial acetic acid, and add appropriate amount of distilled water to give the final volume of 1 L. Autoclave.

10 % (W/V) SDS

Add 100 g sodium dodecyl sulphate (SDS) to 900 ml distilled water, heat to dissolve and adjust volume to 1 L.

0.5 M EDTA [pH8]

Add 186.1 g disodium ethylendiaminetetraacetate (EDTA) to 800 ml distilled water, adjust to pH 8 with 20 g sodium hydroxide (NaOH) pellets and add appropriate amount of distilled water to give the final volume of 1 L. Autoclave.

5 M NaCl

Dissolve 292.2 g sodium chloride (NaCl) in 800 ml distilled water and adjust the final volume to 1 L. Autoclave

50 × Tris-acetate buffer (TAE)

Dissolve 242 g Tris base in 600 ml of distilled water, add 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA, and adjust the volume to 1 L. Autoclave.

1 % Agarose gel (100 mL)

Add 1 g agarose (LE) powder in 100 ml of 1×TAE buffer and heat to dissolve.

Tracking Dye (1 mL)

To the final volume, add

- 30% glycerol
- 30% BTB (bromophenol blue, concentration of 10 mg/ml)
- 40% sterile MiliQ water