Involvement of NAD biosynthesis in the development of stress-induced mutation in *Mesorhizobium loti* strain R7A.

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Abstract

One contentious method by which bacteria deal with a maladapted situation is what has been coined ‘adaptive’, ‘directed’ or ‘stress induced-mutation’. This involves mutants that compensate for some form of sub-lethal stress appearing in large numbers from an apparently non-dividing cell population after a protracted period of time. In *Mesorhizobium loti* this was first observed in the non-symbiotic strains CJ1 and N18. The stress-induced mutation involved the activation of a cryptic chromosomal *dctABD* locus which allowed these strains to utilize succinate as a sole carbon source (Dct^+). In the current study the symbiotic strain R7A, which containsan integrative and conjugative element $ICEMlSym^{R7A}$, was also shown to produce Dct^+ mutants when under stress, when its active *dct* locus on $ICEMlSym^{R7A}$ was deleted. However a derivative of R7A cured of $ICEMlSym^{R7A}$ (strain R7ANS), did not form mutant colonies strongly suggesting that the causative locus was located on $ICEMlSym^{R7A}$. In this work, the causative locus was identified as the *nadQABC* locus, which consists of genes encoding enzymes for the first three steps of the NAD *de novo* biosynthetic pathway (*nadABC*) and an upstream hypothetical ORF encoding a putative nudix hydrolase-like repressor (*nadQ*). Addition of this locus conferred the mutator phenotype on R7ANS. Furthermore, addition of increased concentrations of nicotinic acid, a precursor to NAD biosynthesis via a separate salvage pathway, also conferred the mutator phenotype on R7ANS, whereas increased nicotinic acid led to partial repression of the mutator phenotype in strains that contained the *nadQABC* locus. Mutants of R7A in the *nadA*, *nadB* and *nadC* genes were both nicotinic acid auxotrophs and unable to form Dct^+ colonies without high concentrations of nicotinic acid. Expression data for this locus showed that all three *de novo* genes had significantly higher expression during prolonged incubation when compared to exponential growth, while *nadQ* appeared to be constitutive. Addition of nicotinic acid repressed expression of all three *de novo* genes in a concentration-dependent manner provided that *nadQ*was intact. Mutants in *recA* and *mfd*, genes essential to double-strand-break repair and transcription-coupled repair respectively, exhibited the mutator phenotype in the presence of *nadQABC* or high levels of nicotinic acid. The addition of exogenous catalase did not prevent the mutator phenotype, though it did lead to a small but consistent decrease in Dct^+ colony formation. In conclusion, increased production of NAD was responsible for the mutator phenotype in *M. loti*, through a mechanism independent of either double-strand-break or transcription-coupled repair. This can occur through either *de novo* synthesis or salvage of NAD precursors, though nicotinic acid acted with NadQ as a co-repressor of *de*

novo synthesis, leading to a decrease in mutator phenotype function in NAD prototrophic strains. A model is proposed in which the increase in the concentration of pro-oxidant NAD(H) leads to the formation of reactive oxygen species which in turn elevate the mutation rate due to genotoxic stress.

Acknowledgements

I would like to acknowledge and thank my supervisors, Clive Ronson and John Sullivan, for all the help and guidance they have offered me during the course of my thesis. I would like to extend this to the entire 607 lab for their help and support also.

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List of abbreviations:

ADM – Amplification duplication model

BER – Base excision repair

CMD – cis-mero diploid

Dct^+ - Able to utilize succinate

dNTP – Deoxynucleotide triphospahte

DSB – Double strand break

DSBR – Double strand break repair

DSE – Double strand ends

EDTA – Ethylene diaminetetraacetic acid

GRDM – Glucose defined medium

HMS – Hyper-mutating subpopulation

HR – Homologous recombination

IDM – Insertional duplication mutation

LB – Luria-Bertani

MMR – Mismatch repair

NA – Nicotinic acid

NaMN – Nicotinic acid mononucleotide

NAD – Nicotinamide adenine dinucleotide

NMN – Nicotinamide mononucleotide

NHEJ – Non-homologous end joining

NER – Nucleotide excision repair

PCR – Polymerase chain reaction

RDM – Rhizobial defined medium

RNAP – RNA polymerase

ROS – Reactive oxygen species

SDS – Sodium dodecyl sulphate

SIM – Stress induced mutation or mutagenesis

SRDM – Succinate defined medium

TLS – Translesion synthesis

TAE – Tris-acetate EDTA

TY – Tryptone yeast

UV – Ultra violet light

1.Introduction

Bacteria are constantly faced by a volatile environment, with changes in temperature, ultra violet radiation, pH and nutrient levels that place the organism under physiological stress. As unicellular organisms, they possess few mechanisms with which to change their environment and thus they must undergo internal augmentation to survive rapid change. Because of this, bacterial genomes contain several pathways that allow global changes to metabolism as well as several key features of genomic architecture and replication that allow rapid adaptation and exploitation of novel environments (28).

In regards to altering their genetic constitution to take advantage of new or changing environments, bacteria present us with two accepted strategies: horizontal gene transfer and mutation. Horizontal gene transfer refers to the acquisition of mobilized genetic elements that allow recipient organisms to adapt their physiology and exploit new environments within a relatively short time frame (68). However, horizontal gene transfer relies on the compensatory genetic material being present in the genetic milieu of a given environment, so cannot necessarily solve a novel or unexpected stress. In this sense it is seen more as a key mechanism in rapidly spreading genetic change throughout the population, rather than actually generating that change in itself.

The other main avenue for changing an organism's genetic constitution is that of mutation. Under classical models of mutation/selection, mutations occur at a given rate based on the efficiency of the organism's DNA repair and replication mechanisms as well as the inherent mutability of the genome. This rate of mutation is essentially steady state for any particular organism barring the direct effect of exogenous mutagens (64, 115). Mutation in this model occurs during replication and occurs randomly throughout the genome, thus no particular locus is any more or less susceptible to mutation. If the said mutation presents a particular advantage/disadvantage, then it is quickly fixed/eliminated within the population due to the high division rate experienced by most prokaryotes. This classical model also asserts that the mutation rate of any given organism will reach a certain optimum rate and that a lower rate of mutation is generally advantageous. This is because the majority of all mutations are either deleterious or silent and only a very small percentage are likely to lead to a beneficial change in gene function. Thus, the chances are very high that for any given mutation it will have a

negative effect on cell fitness or viability, meaning that there is selection against high rates of mutation (19-20).

1.1 Stress-Induced Mutagenesis (SIM)

A third paradigm of genetic adaptation in prokaryotes has become increasingly evident, that of stress-induced mutation (SIM) (115). Referred to by a variety of terms such as adaptive, directed, stationary-phase mutagenesis, mutagenesis in aging colonies and starvation-induced mutation (6, 12, 29, 34, 77), SIM involves the appearance of large numbers of mutations that appear to compensate for a particular physiological stress such as nutrient starvation, antibiotics and viral stress. Due to the plethora of terms coined for these particular types of mutation and because all models examined feature induction through one form of stress or another, I will refer to the mechanism in general as SIM. This is not to imply that the mutants are necessarily the direct result of stress (though this may be true in some cases) but rather only that these mutants arise when the cells are under a form of stress. The number of mutants appearing to compensate for each instance of stress is significantly larger than that seen under normal conditions with estimates of up to a 200-fold increase in the rate of mutation (11, 82). Furthermore, the appearance of mutants does not follow a normal Luria-Delbrück distribution. This distribution pattern shows that when cells are plated on selective medium mutants will appear in a random dispersal but at a constant rate, indicating that the mutations existed before plating and that no new mutations arose after (61). Instead SIM shows a Poisson distribution in which positive mutations appear progressively over time suggesting that mutations are arising post-plating, presumably when the cells are no longer growing (12). It is the apparent mutation in a non-growing cell population that contradicts the standard model of mutagenesis as this now excludes errors in replication as a source of mutagenesis, meaning that some other mechanism(s) involved in DNA processing are the cause.

1.1.1 Cairns system

Though several examples of adaptive phenomena in stationary phase were observed as early as the 1950s (87), these findings were largely ignored as the consensus at the time was that spontaneous mutation arose due to errors in replication, thus research into mutation remained largely focused on replicating cells (43). However, this view changed due to what can be viewed as the seminal paper in this field by Cairns *et al*. published in 1988 (13). The initial observation involved an *Escherichia coli* strain FC40,which contained a non-functional fusion between the *lacI* and *lacZ* genes, that was disabled by a +1 frameshift, located on the plasmid F'_{128}. What was unusual was that these *E. coli* cells showed a large number of -1 revertants when grown on media containing lactose as the sole carbon source and that reversion only occurred after several days, from micro-colonies that appeared to be no longer expanding. When this same strain was then grown on media containing either glucose or glucose/lactose as the carbon source, -1 revertants were not observed in significant numbers. This appeared to show that these compensatory mutations were only occurring when the relevant stress was applied; thus the cells were appearing to adapt in direct response to the stress being applied, giving rise to the somewhat misleading term 'directed mutation'(1, 28).

What is unusual and significant about the phenomenon observed becomes apparent once several key factors are taken into account. Firstly, colonies appeared in a linear fashion with about 100 colonies arising over the course of several days from a background cell lawn comprising ~10^8 cells (84). This is inconsistent with the notion that these revertants are the result of spontaneous mutations that arose prior to plating on media as part of normal mutational events occurring during replication. If mutations were occurring during replicative growth prior to plating, then they would be able to utilize lactose as a carbon source immediately and thus not appear over a several day time frame. While pre-plating revertants are present, they have a close to wild-type growth rate and develop colonies within 48 hours (86). Although in other systems heterogeneity of revertants may lead to variation in growth due to levels of fitness advantage from different mutations, this is not the case in the FC40 strain as it requires a specific -1 frameshift to activate LacZ activity and therefore all revertants would be expected to arise within a specific timeframe.

Secondly, there was the issue of revertants only arising when the specific stress (starvation) was applied. If all mutations were arising prior to selection then significant differences in the number of -1 reveratants should not be apparent whether or not lactose was available to

utilize. While it could have been argued that on plates solely containing glucose the number of revertants may be down due to no selective advantage to 'fixing' the mutation in the population, this occurs even when lactose is present but not under starvation conditions, which strongly indicated that starvation or some other stress was required to generate the revertants (12).

One possible explanation for this was that FC40 was a constitutive mutator. This would mean that FC40 mutates at a constantly elevated rate due to some defect in its replication or repair machinery such as a non-functioning mismatch repair (MMR) pathway (34). A strain such as this could be expected to produce a large number of revertants over time due to large numbers of non-specific mutational events constantly occurring. However, a key criterion essentially ruled this out. This is because while -1 revertants did tend to have further random secondary mutations associated with them, which would be indicative of a high mutational rate, they seemed to show no excessive rates of mutation once the selection pressure had been removed. If mutation was occurring constitutively these random mutations would arise regardless of any stress.

Another unusual feature that was observed in the Cairns system was the apparent need for the *lacI-lacZ* fusion to be located on the F' episome. When the construct was relocated to the chromosome only a 2 to 4 fold increase of -1 revertants was observed, compared to the 100 fold increase seen with the episomal copy (82, 86). Such a pronounced difference between two otherwise identical constructs is certainly anomalous and this feature has arguably provided the most fertile source of controversy that subsequently surrounded the Cairns system, which will be expanded upon later.

1.2 Proposed models and mechanisms

Since the initial publication of the 1988 paper, what has now become to be known as the Cairns system has been the subject of intense study and robust debate that among other things has helped expand our understanding of DNA replication, processing and repair as well as cellular responses to stress (40). Because the Cairns system is the best studied of the various SIM systems, I will go into some detail of the various models used to explain it before

discussing some of the other systems that have been observed and the similarities and differences they have to the original Cairns system.

In an attempt to explain the observations of the Cairns system two general models have been proposed, transient hypermutation and the amplification duplication model (ADM), both of which have varying amounts of evidence to support them. The transient hypermutation model postulates that cells under stress enter a state of elevated mutational rates which allows them to achieve a genetic escape from the stress in question. While it is generally accepted that high mutation rates are deleterious to cell survival, there are numerous examples in which elevated mutation rates are selected for. For example, *E. coli* strains that are pathogenic are often constitutive mutators, in that they experience continually high rates of mutation compared to commensal strains (37). The reasons for such a difference become apparent when considering both the effects of mutation and the ecological niche a particular cell line may be expected to inhabit. Mutation, as has been stated, though leading to greater degrees of genetic variation and thus adaptability is also inhibitive at high levels due to the likelihood of deleterious mutations outweighing the chances of an advantageous mutation (97). However, when cells are placed under a significant stress, particularly if growth is arrested, chances of survival in the medium to long term are effectively nil unless there is some form of relieving change in the environment, which is beyond the control of the cell, or there is an adaptive change by the organism itself. As the chances of survival of maladapted cells are nil, any further deleterious mutations will have little or no effect. However any advantageous mutation will be strongly selected for, so in this scenario deleterious mutations provide very little or no negative selection, while advantageous mutations experience strong selection. Thus increased mutation overall is selectively advantageous as it increases the chances of a positive mutation. Pathogenic strains of course must contend with both varying hosts and also shifting attacks from the host immune response; thus an ability to constantly change their genetic makeup will allow a greater chance of producing a mutation that will allow the cell to escape the environmental challenge. Commensal strains on the other hand are present in a far more stable environment and therefore maintaining the status quo holds more selective advantage. This is further reflected in that host-derived *E. coli* strains often have a significant competitive advantage over commensal strains in the initial colonisation of the digestive tract in mice due to their ability to quickly adapt to the new environment, but this competitiveness is reduced over time as deleterious mutations begin to increase once optimum function has been achieved (39).

As can be seen from these examples, high mutations rates can be highly advantageous in certain ecological contexts. However, many bacterial environments are neither entirely stable, nor under constant change, but rather can be characterised by periods of relative stability punctuated by abrupt change or stress in some form (77). Under these conditions one could postulate that a mechanism that facilitates an increase in the rate of mutation during and only during periods of stress would be highly favourable to the organism in question and this is basically the essence of the transient hypermutation model. In the Cairns system the nonlethal stress is in the form of growth arrest due to carbon source starvation. The transient hypermutation model as it applies to the Cairns system can be divided into several models which follow a similar theme but present the exact causes in a slightly different fashion.

The first of these is the formation of mutants via homologous recombination under stress. Much of the basis for this model is the approximately 100-fold difference in the number of Lac^+ revertants depending on whether the allele is located on the F' plasmid or chromosomally (82, 86). For reversion to occur the *lac* construct must also be located close to the origin of conjugal transfer (*oriT*). This contains a *oriT* site that is targeted by the TraI single-strand endonuclease which results in a persistent single strand break at this site (70). The *lacI-lacZ* frameshift construct is also slightly leaky, with 1-2% of the revertant β-galactosidase activity (86). It is posited that this slight leakiness provides enough energy for occasional initiation of replication, though not enough to facilitate actual vegetative growth (30). When the replication machinery reaches the persistent nick at the *oriT* it is stalled. The stalled polymerase complex is removed, causing the recruitment of RecA and the single strand break is resolved to a double strand break (DSB). The recruitment of RecA has several consequences. Firstly, RecA forms a complex with RecB and RecC that is responsible for facilitating homologous recombination as a method of repairing the DSB, basically forming the beginning of the double strand break repair (DSBR) pathway. Secondly, the RecA ssDNA-nucleoprotein filament causes the autoproteolytic cleavage of LexA, a DNA-binding transcriptional repressor of the SOS regulon. This in turn leads to the derepression of about 40 genes involved with cell division regulation and protection against cellular damage, which collectively comprise the SOS regulon or response (34, 51, 90). Of particular note is that the SOS response also induces increased transcription of several DNA polymerases, Pol II (DinA/PolB), Pol IV (DinB) and Pol V (UmuD'C) (51). The UmuD'C polymerase is a dimeric polymerase that is activated by the partial cleavage of the UmuD gene product to yield UmuD'. The autoproteolytic cleavage, like that of LexA, is caused by the activated

RecA (RecA*) (34, 51). UmuD'C is an error-prone polymerase whose primary purpose is to carry out translesion synthesis (TLS) where a replication fork has become stalled due to DNA damage, in particular abasic sites and thymine-thymine dimers (111), and is responsible for the vast majority of mutants in UV-induced mutagenesis (116). However, its role in TLS is restricted to cells undergoing replicative growth so it is unlikely to be a primary contributor during stationary phase (90). The DinA polymerase also serves a specialised repair function and is constitutively expressed, though it is upregulated by both the SOS response and the RpoS stress response. Unlike the two Y family polymerases, it has reasonably high fidelity and a 3' to 5' exonuclease proof-reading function (51) and is therefore unlikely to be a prime cause of SIM though it would appear it has a role in modulating SIM and may be responsible for a minority of mutants (32). Finally, the DinB polymerase is similar to Pol V in that it is also a Y family polymerase involved in TLS and repair. However, DinB functions primarily during stationary phase and furthermore it seems to hold primacy in SIM. This enzyme works in TLS by replacing bases opposite damaged DNA to allow progression of replication, which it does with relatively high fidelity, but it is very error-prone when replicating DNA from an undamaged template strand (33). Despite having differing roles, it appears that all three polymerases have a role during stationary phase as, though they are not essential for survival during stationary phase, deficient mutants in the respective genes have been shown to have a marked decline in competitive fitness at stationary phase when grown against wild-type (116).

The scenario formulated from these observations is that the DSB is repaired via homologous recombination, which under normal conditions would be carried out by a high-fidelity polymerase such as Pol I or Pol III, resulting in very low chances of a mutational event. However, as the SOS response has also been initiated, there is an increase in concentration of DinB, which increases its likelihood of carrying out the DNA replication during this process and thus leading to higher chances of mutations occurring in the region undergoing homologous recombination (70).

There are several pieces of evidence that support this model. Firstly, RecA and the RecBCD and RuvABC complexes are necessary for the formation of revertants (51, 86). The RecBCD complex has both a functional endonuclease and helicase and processes double-strand ends (DSEs) by exposing ssDNA and facilitating the formation of the RecA nucleoprotein filament, thereby initiating homologous recombination. The RuvABC complex has an

endonucleases activity that functions to resolve the Holliday junction which is formed during recombination and must be resolved for the two helices to dissociate (21). The necessity of these two complexes whose sole functions are to facilitate DSBR via recombination indicates that it is not only the SOS-activating properties of RecA that are responsible but also its role in forming the functional pathway for DSBR.

Secondly is the need for the *lac* allele to be co-localized with the *oriT* site, which as stated is thought to have a persistent single-strand break which is proposed to be resolved to a DSB. Evidence for this can be seen in that the number of Lac^+ revertants formed drops in proportion to the distance between the *lac* construct and the *oriT* site. This suggests that as the *lac* construct is moved further away from a DSB, its chances of being included in the mutagenic DSBR decrease. The phenotype can be eliminated by removal of a functional *traI* gene and subsequently rescued by addition of exogenous TraI (70). Virtually the same results are shown when the *lacI-lacZ* construct is colocalized with a *SceI* site which leads to the formation of a persistent DSB after endonuclease action with appearance of revertants increased 1000-fold with the addition of I-SceI (70). These results taken together indicate that the persistent formation of a DSB located close to the *lacI-lacZ* construct is necessary for the normal functioning of the Cairns system.

Thirdly, the presence of an error-prone polymerase is required for the appearance of revertants. Available evidence suggests that DinB is responsible for 85% of revertants, while DinA is not essential but its activity does contribute to the overall phenotype (32). The need and effect of both these polymerases on the Cairns phenotype would strongly suggest that error-prone replication during DSBR is the lead cause in the formation of mutants. Of important note is that while DinB is essential for the formation of revertants during starvation on lactose media, it is not essential for the formation of mutants that occur before plating, hence its mutational activity is restricted to stationary phase and not replicative growth (28).

With the recruitment of error-prone polymerases to DSBR a build-up of errors would begin to appear. If the number of errors were large enough it may have the effect of titrating the ability of the MMR pathway to correct them (or alternatively MMR could be down-regulated during stress) (98). This then raises the question of why this particular phenotype is restricted to a state of starvation or stress. If the persistent nick at the *oriT* site is binding RecA and initiating the SOS response and the following cascade of events just mentioned, then such a process should in theory occur constitutively i.e. during replicative growth, possibly

producing an effect similar to that seen in UV-irradiated cells (90). As this is not the case it would appear that some other event either increases the number of DSBs during stress or further induces the number of mutants associated with its repair. In accord with this, both the RpoS and RpoE sigma factors play a role in the Cairns system (38, 60). RpoS and RpoE are alternate sigma factors of RNA polymerase that bind to differing promoter sequences allowing global changes in gene expression. RpoS is the sigma factor that primarily deals with the stress response in *E. coli*, which affects the expression of about 400 genes and modulates the expression of approximately 10% of the *E. coli* genome during stationary phase (18). Though RpoS is expressed and regulates some genes during exponential growth, its activity is greatly increased during stationary phase due to the removal of proteolytic repression, which leads to global changes in gene expression. Of particular note is that RpoS leads to a roughly 2-fold increase in the expression of DinB. While this is small compared to the 10-fold increase caused by the SOS response, combined they may cause enough DinB-induced error to titrate the MMR system.

RpoE on the other hand modulates the cell's response to the stress incurred by incorrectly folded extracytoplasmic proteins. Like RpoS, it shows constitutive expression that is vital for cell viability but, when activated, leads to the modulation of about 200 genes from a wide variety of metabolic processes. How exactly it interacts with the Cairns system is unclear but its removal leads to a decrease in the number of revertants. This decrease can be rescued by the addition of I-SceI endonuclease which strongly suggests that RpoE functions by facilitating the formation of DSBs, thereby increasing the potential for the formation of revertants. However, how exactly RpoE causes this is still largely unknown (38).

From all these lines of evidence it would appear that the model put forth has been coupled with two different stress response systems to switch DSBR from a high-fidelity process under growth conditions to an error-prone mutagenic process when under stress. It would also seem that a third stress response has also been recruited, either to increase the formation of DSBs from SSBs or for a generally promotive role in DSB formation.

The advantage of this model is how well it resolves the observations of the Cairns system. It does this by sequestering the mutagenic process to both a particular period of time and also a specific area of the genome. By being coupled with the stress response high mutation rates will only be experienced transiently while the cells are under stress conditions, in this case carbon source starvation. When the cell has escaped this stress activation of stress factors will

cease and DSBR will return to a high-fidelity form, which explains why Lac^+ revertants seem to show no increased rates of general mutation. With mutation occurring with the formation of DSB, only loci located close to a persistent lesion will form mutants via this pathway, lowering the chances of random deleterious mutations occurring in the rest of the genome.

Whether this mechanism is a deliberate regulated strategy to modulate evolutionary rates during periodic stress as opposed to essentially being a laboratory artefact seems uncertain. While the coupling with the stress response would argue for it, the need for loci to be co-localized with a persistent lesion means that the chances of a beneficial escape mutation occurring are greatly reduced. Loci that have the potential to alleviate a stress would not be expected to have any greater chance of being adjacent to a persistent lesion than any other loci as the assortment of loci and persistent lesions would be expected to be random. So in terms of a general mechanism it would be unlikely to have a great deal of relevance in term of bacterial evolution. This is largely because it has been formulated using the particular criteria and circumstances present in the FC40 strain. However, it could act as more general mechanism but would require the formation of lesions across the episome in a far more irregular and random manner, which would remove the need for relying on the particular nature of local genomic architecture.

This model unfortunately does not explain why within a subpopulation comprising about 10% of revertants and 0.06% of the total cell population, there are indeed significant numbers of secondary mutations occurring on both the F' plasmid and on the chromosome (82). The second model proposed which seeks to explain this feature of the Cairns system through elevated mutation is that of the hyper-mutating subpopulation (HMS). This model essentially holds that most if not all Lac^+ revertants observed are the result of a small subset of cells that experience elevated rates of general mutation in comparison to surrounding cells during stress and again is described as transient. The way in which mutants arise may either be through the DSBR model described above or through an independent and distinct mechanism and there are varying amounts of evidence to support both arguments.

When tested for auxotrophy of various unselected reporter genes, Lac^+ revertants have 50 times as many secondary mutations as occur in the Lac^- background (40). This would be unexpected if all cells under stress experienced general mutagenesis, as the revertants having escaped growth-limiting stress would return to a low mutagenic state, while the Lac^- background would still accumulate secondary mutations while waiting for an escape

mutation. The observation therefore strongly suggests that only a small minority of the cells enter a state of elevated mutation through some as yet unknown mechanism. However, this assumption becomes less clear when the clustering of mutations within the revertant population is considered. Most Lac^+ revertants are in fact free of secondary mutations, and screening for non-selected mutations show that they occur at a significantly higher frequency in cells already containing one or more secondary mutations, than in otherwise non-mutant Lac^+ revertants (82). This suggests most Lac^+ revertants do not experience significant secondary mutation and that this is restricted to a small subset of cells that contain multiple mutations.

Though the presence of the HMS is fairly well accepted, how substantial its role is in the Cairns system is still a matter of debate. Some argue it represents only a small minority of cells unrelated to the main phenotype (82), while others argue that it is responsible for the majority of mutations and is clearly related to or part of the DSBR model mentioned above (40). The types of mutation leading to revertants in cells marked as part of the HMS are virtually identical to those in a typical Lac^+ revertant. The majority of these mutations seem to be in the form of a single deletion in nucleotide repeats to reverse the frameshift. This is in stark contrast to generation-dependent Lac^+ revertants which show a far more heterogeneous spectrum of mutations, such as 4 and 8-bp deletions, 2-bp additions as well as larger more significant deletions and insertions. Furthermore, addition of I-SceI, which increases both the number of DSBs and subsequent Lac^+ revertants, does not lead to a change in the proportion of apparent HMS cells in the revertant population. If HMS were an entirely independent mechanism then the number of HMS-derived colonies would remain constant while the overall numbers of revertants would increase, thus decreasing the proportion of HMS in relation the revertant population a whole. As this is not the case and given the homogeneity of the mutational spectrum encountered, these observations suggest that DSBR coupled mutagenesis and the HMS phenomenon are either closely related or one in the same (40).

By definition, HMS functions as a stochastic process, in which hypermutability is activated through unknown means in only a small subset of cells. One possible mechanism is that cells may have to reach a certain threshold of both SOS and RpoS induction to reach a state of hypermutability, and differences between cells as well as spontaneous initiation of SOS could explain a differential effect (40). However, it has also been shown that strains that constitutively express DinB at SOS-induced levels show no increase in Lac^+ revertants (33).

If the DSBR and HMS were one in the same, then one would expect a dramatic increase in the number of Lac^+ revertants as the entire population are now effectively hypermutators. These results taken together, though initially contradictory, would suggest that the two processes are related in that the HMS arises through low fidelity DSBR but not all Lac^+ revertants arising through low fidelity DSBR are part of the HMS. The ability to resolve the proportion of Lac^+ revertants encompassed by the HMS is problematic as they seem to show a shared origin to DSBR Lac^+ revertants and disentangling the two with any form of accuracy when there are only subtle differences is difficult. Thus it can only really be said with any form of certainty that firstly there is a subpopulation of hypermutating cells and secondly that this subpopulation only forms a minority of the Lac^+ revertants seen in the Cairns system, though the size and significance of this minority is still undetermined. The mechanism through which the HMS may form is also still undefined. The ADM model does offer one alternative explanation, and I will elaborate further on that in the following section.

The idea of stress-induced mutagenesis or even variable regulation of mutagenesis as illustrated by the Cairns system though now far from new has run against the classical model of replication-based mutagenesis and as such is not without its critics and rebuttals. Critics maintain that raising the level of mutagenesis is not desirable or even possible at levels proposed by SIM models. More importantly they assert it is not necessary to resort to an elevated mutational rate to explain any feature of the Cairns system and that the phenotype can be fully explained via workings of conventionally understood processes (86). Critics cite a number of unresolved issues within the Cairns system such as either the lack of, or inconsistent distribution of, secondary mutations, the need for improbably high mutation rates,lack of reversion when the *lac* construct is located on the chromosome and the apparent need of a certain base-line leakiness in expression of the target gene (86, 94, 115).

One of the main cruxes in the argument against SIM is that only a very small number of mutations ever have any beneficial effect and that the majority of these tend to be small phenotypic events in any case. Thus, the likelihood of achieving a major phenotypic change to overcome a short-term stress within a generational timeframe is infinitesimally small compared with the far higher likelihood that the mutation will at least subtract from the fitness of the organism if not act lethally (1, 84). This is precisely why bacteria have such a diverse array of repair systems in place to protect against mutation and why even the fundamental properties of DNA as an information system are arranged to prevent the effectof

mutation on phenotype, such as the arrangement of codon translation to minimise the inevitable tendency of DNA to become GC rich (57).

Moreover, they stipulate that even if such a mechanism did exist in bacteria, the level of mutation needed by the Cairns system to achieve the apparent reversion rate is astronomical. In the homologous recombination-coupled DSBR (HR-DSBR) mutagenesis model the ~100 revertants that arise from the 10^8 cells plated would require a roughly 100-fold increase in mutation. The HMS model on the other hand (taken to its extreme where it is responsible for all revertants) requires that these 100 revertants arise from just 0.1% of cells plated which amounts to a 10^5 fold increase in the rate of mutation, a figure that is hard to achieve even with harshly mutagenic treatments (85).

The reason for this assertion is the number of requirements and inconsistencies peculiar to the Cairns system. Though I have already mentioned some of these in passing, I will review them here again to present their relevance to the following model. Probably the most important criticism is the need for soft selection in the Cairns system. Not only is the Cairns system a permissive system (which is necessary if one wishes to observe post-plating mutagenesis in any form) but requires a small leakiness of function from the *lacI-lacZ* construct, with reversion greatly reduced when a similar but completely non-functional construct is introduced (2). In the SIM model this is due to the small input of energy provided by leakiness that allows initiation of replication but not completion. However here it is proposed that this leakiness does in fact input enough energy to allow growth, albeit slowly, especially when coupled with other factors (86).The need for the construct to be placed episomally is also seen as important as is the general lack of secondary mutation in the rest of the genome in the vast majority of cells, which argues strongly against an increase in mutagenicity.

Based upon these criticisms a second alternative theory has been proposed, the ADM model. The ADM model asserts that the Lac^+ revertants encountered in the Cairns system are not the result of an increased mutation rate but are in fact due to amplifications of the *lac* region on the F' episome that are already present in a heterogeneous population prior to plating. Amplifications are the most commonly occurring form of mutation. They occur at such high rates that they are maintained at an equilibrium in natural populations, despite negative selection against them (1, 86). Within the growing culture of cells there will be a small heterogeneous subpopulation of cells carrying varying numbers (usually a duplication) of the *lacI-lacZ* construct which have been generated through amplification. When these are plated

out, the leakiness of the *lacI-lacZ* construct means that cell carrying two or more copies may be able to generate enough energy to establish slow vegetative growth and more specifically complete replication, with additional copies offering greater potential for growth. As the cells are now replicating this presents an avenue for the formation of even further amplifications of this locus, which will experience positive selection as they will confer a greater capacity to exploit lactose, while spontaneous loss of the amplification will be selected against (1). Apart from increasing the cells fitness on lactose media, the increase in the numbers of the *lacI-lacZ* construct also offers a far larger target for a mutation to occur, as there are now multiple copies of the gene in which a mutation could take place. When a true Lac^+ revertant is generated at one of the multiple loci now available, the selective pressure reverses and the amplifications are steadily lost as they are unstable and there is no longer a selective pressure to maintain them. In the course of this resolution of amplifications, some of the new Lac^+ revertants will be lost as there is no directed preference as to which copy of the locus is retained. However, strong selective pressure should maintain Lac^+ revertants in the population and once the amplifications have resolved themselves the frameshift mutation should become fixed (108). Because the number of amplifications both in the original culture and especially after selection is variable between cells, this in turn leads to variable growth rates that explain the Poisson distribution exhibited in the Cairns system (86). It is important to note that because amplifications are proposed to be small-effect mutations that skirt the edge of the selective pressure, they require a permissive pressure such as starvation which allows incremental escape rather than a rigid selective pressure with strict positive and negative phenotypes such as those seen in antibiotic resistance (84).

The amplification model also offers an explanation for the more general mutagenesis seen in the HMS. A copy of the *dinB* gene in the FC40 strain is also located on the F' plasmid ~16kb from the *lacI-lacZ* construct. The amplification model proposes that due to the small distance in genomic space between the two loci a number of *lac* amplified strains will also carry a co-amplification of the *dinB* gene. From here this amplification of *dinB* acts in a way similar to that described in the SIM models in that the increase in DinB leads to error-prone replication of DNA which overloads the ability of the MMR system to repair (94).

There are several features of the system and lines of experimental evidence that support the amplification model, though as in the two SIM models the evidence is mixed and open to interpretation. In terms of some of the established genetic requirements of the Cairns system,

most of the genes found to be associated with it such as RecA, RecB, RecC and RuvABC are all required for homologous recombination. While in the SIM model, this is explained by low-fidelity homologous recombination in the amplification model it is explained because amplification occurs through unequal crossover during homologous recombination (1, 108).

The need for the *lac* construct to be located on a plasmid rather than chromosomally is explained by the repeat sequences flanking the *lac* construct on the plasmid and also the intrinsic property of plasmids that help facilitate recombination and thus amplification. The location of an *oriT* site adjacent to the *lac* construct also serves by increasing the formation of DSB and DSEs. DSBs formed by TraI and ISec-I would merely serve to increase the rate of homologous recombination and thus the likelihood of an amplification occurring at that particular locus (54, 96).

Apart from this indirect support for amplification, there is also direct experimental evidence that amplification is required for the formation of revertant colonies. In one experiment a Tn10 transposon containing a *tetA* gene was placed on the F' plasmid at increasing distances from the *lac* construct (from 5 to 104 kb) (47). The *tetA* gene acts as an efflux pump and when present in a single copy cells are tetracycline resistant (Tc^r). However, when multiple copies are present, it confers tetracycline sensitivity (Tc^s) as over-production of the efflux pump leads to a loss of membrane potential (23). Thus in this configuration if cells are grown with tetracycline any amplification of *tetA* will result in a lethal phenotype. When these strains were grown on lactose plates containing tetracycline, there was a marked decrease in the number of Lac^+ revertants produced in rough proportion to the distance between *lacI-lacZ* and *tetA* (as the distance between the two loci increases so too does the number of revertants) (47). This has been taken to demonstrate that when *tetA* is located close to the *lac* construct the chances of them being coamplified are greatly increased leading to a strong nonpermissive selection against amplification of the *lac* construct. As *tetA* is moved further away, the chances of coamplification decrease and therefore so does selection against amplification of *lacI-lacZ*. That the number of Lac^+ revertants decreases significantly when there is a strong selective pressure against *lacI-lacZ* amplification strongly supports the hypothesis that these revertants arise through amplification. The presence of cells containing amplifications have also been detected and cells exhibiting amplifications can reach as high as 40% in revertants appearing after 5 days (71).

In regards to the coamplification of *dinB*, it appears to be amplified in about 20% of cells (95), though this is slightly higher than the generally accepted 10% attributed to HMS (86). Furthermore, when the location of *dinB* is changed to either a different location on the F' plasmid, or placed *intrans* such as on the chromosome or on a separate high copy number plasmid, then the number of revertants that can be accounted for by HMS is greatly reduced (94). This shows that DinB must be closely located in *cis* to *lacI-lacZ* for the HMS to form, supporting the hypothesis that DinB is inadvertently gaining positive selection via coamplification with *lacI-lacZ*, resulting in increased rates of mutation.

The amplification model however is not without its detractors. In particular are the several established criteria that it does not account for as well as a general lack of amplification at large. The need for DinB and RpoS is not really accounted for by the amplification model. While it has been suggested that this merely represents the removal of the HMS, this does not reconcile with the actual reduction in revertants. As these two factors have no role in the formation of amplifications, it would seem difficult to reconcile their involvement with a model that has amplification as the source of the majority of Lac^+ revertants. Furthermore, while there is a significant number of revertant colonies after 5 days on plates, there does not appear to be significant numbers of amplifications before this. As the loss of *lacI-lacZ* amplifications in true Lac^+ revertants should occur in an essentially random manner, there should be no significant difference in detectable amplified cells at any particular time point. That they only appear in significant numbers after day 5 would indicate that the cause is more complex than the amplification model would suggest. Also even when amplifications are detected in significant numbers, they still do not appear as the majority of revertants encountered.

The number of amplifications required for the activating +1 frameshift to occur at such a high rate without the need for an increased mutation rate has also been called into question. If the figure of one activating mutation occurring per 10^8 *lacI-lacZ* constructs, which is roughly the normal rate of mutation, then a colony of 10^4 cells would require that each cell within the colony have an average of 100 amplification of the *lac* construct (98). This seems like an improbable number presuming that the amplifications occurring pre-selection are fairly few, and that the number of divisions that have taken place are insufficient to produce such high level. In this same vein, it is also unclear as to why there are so few Lac^+ revertants when the *lac* construct is placed chromosomally. Even flanking repeats added to the chromosomal

construct do not resurrect the mutator phenotype (96). The apparent need for a functional TraI undermines the basic premise of the amplification model in which duplications and amplifications are common to begin with.

Rebuttal has also been made of the supposed coamplification of *tetA* when placed closely in *cis* with *lac*. For example the percentage of Lac^+ revertants that are inhibited by the induction of *tetA* is the same no matter the distance between the two loci (though the overall numbers of Lac^+ revertants is reduced as the distance reduces), despite the fact that at the two greatest distances used, 99 kb and 104 kb, the chances of coamplifying with *lac* are low as most amplifications are between 10 to 40 kb in length (98).

In general the amplification model has proved inadequate to fully explain the Cairns system phenotype and it would appear that the concept of transient mutagenesis (by whatever mean) currently enjoys greater support, especially in light of data collected from other taxa (34).

The three models discussed here that attempt to solve the Cairns system are not in themselves mutually exclusive and it is highly likely that all three phenomena take place. What is still uncertain is to what proportion each solution holds in the production of revertants.

1.3 SIM in other systems

Since the release of the Cairns 1988 paper, SIM has been observed in a wide variety of conditions and contexts such as antibiotic resistance, nutrient limitation and reversion to amino acid prototrophy. These studies have been carried out in both the original organism, *E. coli*, as well as a variety of other organisms and have revealed a host of other factors and mechanisms involved.

The appearance of mutants in ageing colonies of *E. coli* is not restricted solely to the FC40 strain. Examination of some 787 natural *E. coli* isolates found that over 80% of them showed an increased rate of mutagenesis in ageing colonies (MAC) (6), attesting to the widespread use of this SIM in nature.Aside from *E. coli*, SIM has been observed in a variety of other bacterial species such as *Salmonella enterica*, *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Bacillius subtilis* to name a few (16, 52, 86). Investigations into these other

bacterial systems have further reinforced the ubiquitous nature of SIM as well as provided much insight into the variability of mechanisms employed to generate SIM.

SIM in *B. subtilis* has received extensive study as it seems to operate under different mechanisms to those identified in the Cairns system. *B. subtilis* is a Gram-positive bacterium found in soil (77) and in this regard is of relevance as it is adapted to similar environmental conditions as experienced by the organism used in the present study. The first major difference between *E. coli* and *B. subtilis* is that RecA is not a requirement in *B. subtilis* (103). This renders the role of recombination defunct in this SIM system which rules out the HR-DSBR and ADM models suggested for the Cairns system. The induction of SOS is also precluded to a certain extent, though unlike *E. coli*, *B. subtilis* has the potential for RecA-independent induction of SOS (103). Furthermore, the alternate sigma factor σ^B, which is a homologue of RpoS in *E. coli*, does not seem to have any effect on SIM in *B. subtilis*(103). While *B. subtilis* shows some overlap with *E. coli* in that it requires a Y family DNA polymerase which shares homology with *E. coli* DinB, it appears to operate in a manner independent of both RecA-mediated SOS or σ^B induction (104).

One unique feature of SIM in *B. subtilis* is the involvement of ComA and ComK, transcription factors that regulate the development of competence. While unlikely to have a directly mutagenic role, they probably play a role in regulating the genes involved in SIM. While competence may initially seem unrelated to processes involved in SIM, the competence pathway does offer a mechanism by which cells can form a differentiated subpopulation, which is of vital importance to stochastic processes such as those suggested in the HMS model (103).

Another SIM mechanism that *B. subtilis* has proved particularly helpful in illuminating is that of transcription-mediated mutagenesis. The process of transcription has many properties that are readily amenable to SIM. It provides an opportunity for DNA processing during stationary phase, confines action to only a portion of the genome and offers the potential for feedback. The build-up of mutagenic transcripts may provide a process by which cells have a selective bias towards mutations in transcribed genes. The transcription repair factor Mfd is needed for efficient SIM in *B. subtilis*, possibly through allowing RNA polymerase to bypass non-distortive lesions and transcription to proceed (83). While Mfd is not involved in the Cairns system, the transcription elongation factor NusA has been shown to be involved in SIM by recruiting DinB to stalled transcription complexes (15), demonstrating that

transcription-mediated mutagenesis may also be relevant in *E. coli*. This is further demonstrated in that the greater the induction of a locus the higher its rate of reversion (72), suggesting that high transcript levels or at least access to transcription are key to SIM in *B. subtilis*.

Observations of elevated mutation rates under stress have also been made with eukaryotic organisms. Easily the most studied eukaryotic organism in this field is the budding yeast *Saccharomyces cerevisiae*, due to its ubiquity as a model organism and the fact that it is unicellular and is amenable to experimental assays comparable to those used for bacteria.Investigations into adaptive mutation in *S. cerevisiae* stemmed directly from the finding revealed in the Cairns paper. When assessing whether adaptive mutation occurs in *S. cerevisiae* several differences must be noted between it and *E. coli*. The fact that it is eukaryotic has several important implications. Firstly, it has complex cellular structures such as organelles, and in particular a nucleus which means genetic material is not in direct contact with the cytosol and is thus less readily affected directly by the state of the cell as a whole. Secondly, yeast chromosomes exist in the form of chromatin which could add some protective effects against DNA damage and change the specifics of DNA interactions. Thirdly, while eukaryotes generally have specific DNA repair functions, some of these appear homologous to systems seen in prokaryotes such as MMR, while others are merely analogous such as base excision repair (BER) (45). Basic differences in how these proteins interact with DNA and their regulation could have profound effects on how adaptive mutation might function in the respective organisms. Lastly, *S. cerevisiae* can exist as either a haploid or diploid organism. This potential for two copies of a gene to be present in some cells has particular implications to both the HR-DSBR and ADM models as well as more general implications in regards to replication and DNA processing mechanisms.

The mechanisms operating in *S. cerevisiae* do show some similarities to those seen in prokaryotic systems, in particular the use of DNA polymerases involved in TLS. Rev1 and Rev3/Pol ζ are both enzymes involved in TLS in *S. cerevisiae* and are required for SIM to take place. Rev1 is a member of the Y family DNA polymerases, while Rev3 is a TLS polymerase specific to eukaryotes (45). Rev3 accounts for ~30% of SIM revertants in *S. cerevisiae* (46). However, while TLS polymerases are required for SIM, they appear to operate via a different mechanism than that suggested in *E. coli* in that proteins required to

repair DSBs via HR and single-strand annealing (SSA, a method specific to eukaryotes) appear to have no or little effect on SIM in *S. cerevisiae* (45).

DSBs do appear to have a major role in *S. cerevisiae* but appear to form mutations via a mechanism that is common in eukaryotes (though less common in prokaryotes and via a far more compact version), non-homologous end joining (NHEJ) (41, 45, 69). NHEJ is a mechanism to join DSEs in eukaryotes that requires no or very limited (micro-) homology between the two ends. Deletions of genes essential to NHEJ result in a 50% decrease in revertants in *S. cerevisiae* suggesting that NHEJ is a major mechanism in SIM formation (46). Furthermore, it would appear that there may be competition with HR to repair DSBs in diploid cells, adding complexity to the mechanism. The use of this system in SIM does not appear to have an analogue in prokaryotes and thus would imply that this is an independent evolution of an SIM mechanism in eukaryotes. While DSBs are repaired in the minority in *S. cerevisiae* (except possibly under stress as this model suggests), this is not the case in other eukaryotes such as mammalian cells, and thus such a mechanism could have quite profound implications. This model also suggests that the number of DSBs are increased during stress though the mechanism is still as yet unknown (45). It must also be noted that the contribution of TLS polymerase-induced SIM and NHEJ are cumulative rather that complementary, suggesting that two (at least) independent mechanisms for SIM operate in *S. cerevisiae.* This raises the possibility of variable initiation of SIM with stochastic properties important to potential HMS (46).

That SIM is also observed in a eukaryotic organism has implications beyond that of the evolution of such a mechanism. In multicellular organisms the majority of cells are held in arrest which could be loosely described as analogous to the growth inhibition provided by starvation, though in this case mutation past cell cycle check points is required to escape the ‘stress’. Thus, if such mechanisms are also present in higher eukaryotes they could have important implication for both the formation and progression of cancer (11).

Overall the collection of further data from *E. coli* and a diverse range of other taxa have shown that SIM is both a real and common adaptive strategy. Whether the mechanism is a shared one (i.e. conserved) or has been independently evolved in each case is open for debate and requires further elucidation of the mechanisms involved. However, it clearly demonstrates that this phenotype is not restricted to the Cairns system and renders some of the underlying deficits in that system as moot points.

1.4 Current study

1.4.1 *Mesorhizobium loti*

The bacterial species used in the current study is the rhizobium species *Mesorhizobium loti*. Rhizobia represent a group of soil bacteria that are of particular ecological and agricultural importance as they are able to form symbiotic root nodules with leguminous plants, allowing the fixation of nitrogen. Rhizobia are a diverse and paraphyletic group of both alpha and beta proteobacteria spread across several genera. Thus it appears that the various rhizobial taxa split long ago and that symbiosis has largely been conferred through horizontal gene transfer, evidenced by the common location of symbiosis genes on large plasmids or symbiosis islands, and facilitated by the saprophytic environment of soil bacteria that enable efficient gene transfer (62, 102).

M. loti is a microsymbiont of several species of the *Lotus* genus including *L. japonicus* and *L. corniculatus*(102). The particular strain of *M. loti* used in these experiments is unusual among rhizobia in that the symbiosis genes, which are encoded on accessory replicons in most taxa, are located chromosomally, albeit on an integrated mobile genetic element. This mobile genetic element is a ~500 kb sized region encoding 414 predicted genes, many concerned with symbiosis but others, such as the biosynthetic operons of the vitamins biotin, thiamine and nicotinate, and those involved in metabolic processes and transport, may indirectly augment survival within the rhizosphere (99). In addition, there are a number of hypothetical gene clusters with as of yet unknown function (102). This mobile genetic element was coined the ‘symbiosis island’ due largely to its similarities to pathogenicity islands seen in pathogenic Gram-negative bacteria, including integrating via a P4 class integrase. The symbiosis island appears to behave in a way analogous to both a large transposon (such as integration), and a conjugative plasmid employing a similar method of transfer (75). It is thus an integrative and conjugative element (ICE) (114) and was recently renamed ICEMlSym^{R7A} (74).

1.4.2 *dct* locus

The *dct* locusis of central importance to this study. This locus allows for the transport of the C_4-dicarboxylates succinate, fumarate and malate and is essential for the ability to fix nitrogen in most species of symbiotic bacteria. The *dct* locus consists of three genes that encode a permease (*dctA*) which allows the entry and subsequent utilization of succinate and a two-component regulatory system consisting of a trans-membrane sensor kinase (*dctB*) and a cytoplasmic transcriptional regulator (*dctD*), which in turn activates the transcription of *dctA* (117).

1.4.3 Previous work and discovery of the mutator phenotype

An SIM phenotype involving mutation to succinate utilisation was initially observed in the non-symbiotic *Mesorhizobium* strains CJ1 and N18. The mutator phenotype involved the activation of a cryptic chromosomal *dctABD* locus. These genes are termed cryptic as although they appear intact, most non-symbiotic strains of mesorhizobia are not able to grow on succinate as a carbon source. In contrast, symbiotic strains can grow on succinate because ICE*Ml*Sym^{R7A} also encodes a copy of the *dctABD* genes which is fully functional (102, 112). When grown with succinate as the sole carbon source, most non-symbiotic mesorhizobial strains form micro-colonies that arise due to growth on impurities in the medium and pre-existing cellular reserves, but growth is soon stalled due to starvation. With most such strains, no succinate- positive (Dct$^+$) colonies arise, indicating that the spontaneous frequency of mutation to Dct$^+$ is very low. When strain CJ1 is plated on succinate media, it also initially shows the same micro-colony formation; however, after several days, large numbers of Dct$^+$ colonies begin to appear, far exceeding that of the other strains (Figure 1.1). Work by Weaver (112) showed that the difference between the CJ1 and other similar strains was due to the presence of an indigenous plasmid and cloned the region of the plasmid that appeared to be responsible into a broad-host range vector. When this plasmid pJW5 was placed in CJ3, which was previously Dct$^-$, it caused the mutator phenotype in this strain also. The Dct$^+$mutants that arose in the presence of pJW5 were shown to be caused by point mutations in either of the cryptic chromosomal *dctB* or *dctD* genes (112). This finding is unique in the literature of SIM as most genes identified as being responsible for SIM in other systems

generally result from the deletion of pre-existing native genes, usually involved in DNA repair. This is

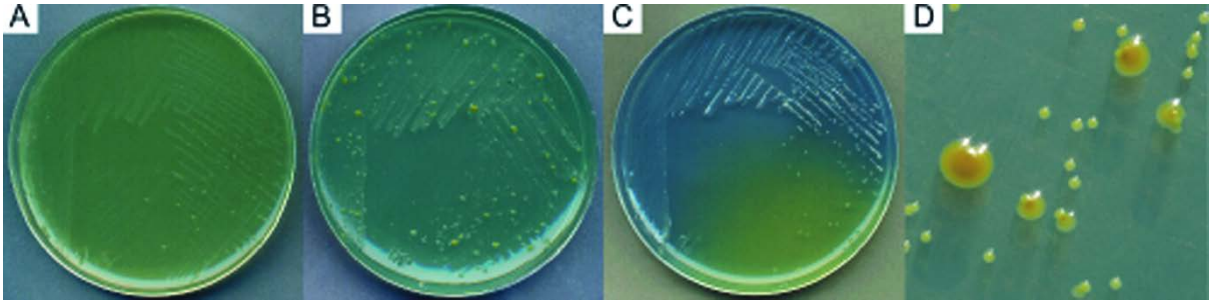


Figure1.1: Dct phenotypes of non-symbiotic mesorhizobia following growth on SRDM. (A) The Dct^- phenotype displayed by CJ2, CJ3, CJ4, CJ6. (B) Dct mutator phenotype displayed by CJ1 and N18. (C) Dct^+ phenotype displayed by CJ5 and CJ7. (D) SRDM plate of CJ1 showing both Dct^- micro-colonies (smaller colonies) and Dct^+ (bigger and irregular in shape) colonies. Photos A, B and D were taken 28 days post-inoculation and photo C seven days post-inoculation. (Figure provided courtesy of Dr J. Weaver).

the first report as far as I can ascertain from the literature that direct addition of an exogenous element to a naive organism conferred a SIM phenotype (at least when the target gene is chromosomal).

Weaver sequenced and annotated the insert in pJW5 (Genbank entry EF618553.1; (Figure1.2)) and attempted to identify the genes on the plasmid responsible for the mutator phenotype. This work led to the suggestion that a pair of toxin-antitoxin loci, *hipB1A1* and *hipA2B2*, were involved. Hence the hypothesis at the beginning of the current study was that the adaptor phenotype was due to the action of a toxin-antitoxin locus. It was proposed that a sub-population of cells under stress enter a transient non- or very-slow-growing state mediated by HipA where they undergo hypermutation to generate adaptive mutants. Subsequent studies carried out by others in the Ronson laboratory during the first year of the current study showed that the *hip* loci were not involved and hence that the hypothesis was incorrect.

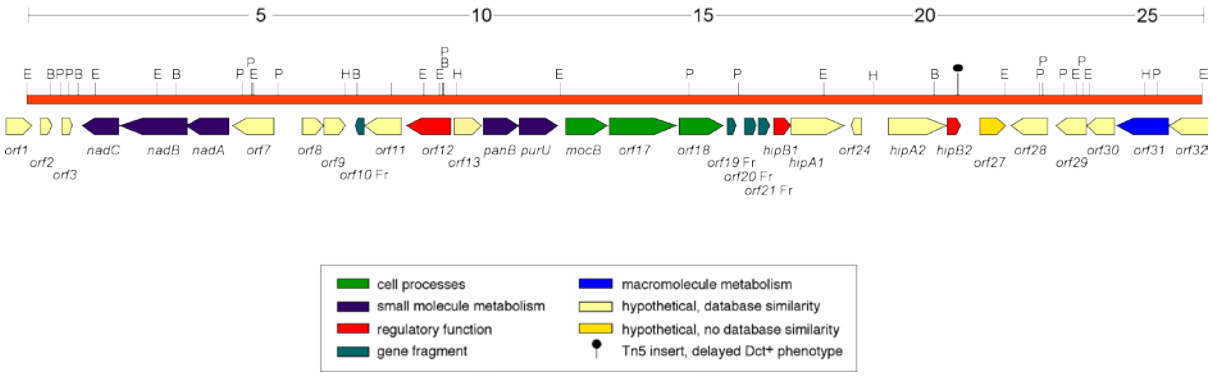


Figure 1.2: Map of the pJW5 insert. (Figure provided courtesy of Dr J. Weaver).

A second *M. loti* strain was also identified as displaying an adaptor phenotype, R7A. R7A is a symbiotic strain and so contains ICE*Ml*Sym^{R7A} that encodes a set of functional *dct* genes that allow the strain to grow on succinate. However, when the symbiosis island set of *dct* genes was deleted, the resultant strain R7AΔ*dctABD*^{Sym} (hereafter called R7AΔ*dct*) showed the adaptor phenotype, giving rise to a high a number of Dct^{+} colonies, presumably from mutational activation of the cryptic chromosomal *dct* locus. In contrast, a derivative of R7A cured of ICE*Ml*Sym^{R7A}, strain R7ANS, did not show the adaptor phenotype, suggesting that a locus on ICE*Ml*Sym^{R7A} was responsible for the phenotype, inducing adaptive activation of the chromosomal *dct* locus.

The overall aims of this study were to characterise the mutator phenotype conferred by the R7A symbiosis island, locate the specific locus or loci responsible for the phenotype, and also determine mechanism(s) underlying the phenotype. In the course of this study the loci conferring the mutator phenotype were identified as genes responsible for the first three steps of the NAD *de novo* biosynthetic pathway and aspects of its regulation were examined. This study also showed that the involvement of either DSBR- or transcription-mediated mutagenesis is unlikely to be of relevance to the mutator phenotype in *M. loti*. Finally I propose a tentative model in which increased NAD(H) levels lead to increased levels of reactive oxygen species under stress to create this adaptor phenotype.

2. Methods and Materials

2.1 Media and Growth Conditions

E. coli was grown in either 5-ml Luria Bertani (LB) (63) or tryptone yeast (TY) (4) broths, or on LB agar. *M. loti* was grown on either 0.4% glucose rhizobium defined medium (GRDM) (81) or 0.4% succinate rhizobium defined medium (SRDM) (80) and liquid cultures were grown in 5-mL broths of either TY or GRDM. *M. loti* was incubated at 28°C while *E .coli* was incubated at 37°C. All liquid cultures were incubated in shakers. All solid RDM media were prepared with Noble agar (Coast Biologicals Ltd), unless otherwise stated. All RDM media contained 1 µg/ml of nicotinic acid as part of the normal vitamin compliment unless otherwise stated. In cases where the concentration of nicotinic acid was increased, addition of a 10 mg/ml stock solution filter sterilized in MilliQ water was used.

2.2 Bacterial strains

The bacterial strains used in this study are described in Table 2.1.

2.3 Plasmids

Plasmids used in this study are described in Table 2.2.

2.4 Primers

Primers used in this study are described in Table 2.3

2.5 Antibiotics

Antibiotics used in this study are described in Table 2.4 and were purchased from Sigma. Antibiotics were dissolved in 100% methanol or dissolved and filter sterilized through a 0.45-µm syringe filter in MilliQ water, as appropriate.

Table 2.1: Bacterial strains used in this study.

Strain	**Description**	**Source**
M. loti		
R7A	Symbiotic *Mesorhizobium* isolate	(101)
R7ANS	Nonsymbiotic derivative of R7A cured of ICE*Ml*Sym^{R7A}	(75)
R7ANSΔ*recA*	Δ*recA* markerless deletion mutant of R7ANS	Previous study
R7ANSΔ*mfd*	Δ*mfd* markerless deletion mutant of R7ANS	Previous study
R7AΔ*dctA*	R7A symbiosis island Δ*dctA::Ωkan* marker exchange mutant	(112)
R7AΔ*dctAB*	R7A symbiosis island Δ*dctAB::Ωkan* marker exchange mutant	(112)
R7AΔ*dctB*	R7A symbiosis island Δ*dctB::Ωkan* marker exchange mutant	(112)
R7AΔ*dctD*	R7A symbiosis island Δ*dctD::Ωkan* marker exchange mutant	(112)
R7AΔ*dct*	R7A symbiosis island Δ*dctABD::Ωkan* marker exchange mutant	(112)
R7AΔ*dct nadA::pFUS2*	*nadA::lacZ* IDM mutant of R7AΔ*dct* created by insertion of pFUS2. Gm^{R}	This study
R7AΔ*dct nadB::pFUS2*	*nadB::lacZ* IDM mutant of R7AΔ*dct* created by insertion of pFUS2. Gm^{R}	This study
R7AΔ*dct nadC::pFUS2*	*nadC::lacZ* IDM mutant of R7AΔ*dct* created by insertion of pFUS2. Gm^{R}	This study
R7AΔ*dct nadACMD::pFUS2*	R7AΔ*dctABD nadACMD::pFUS2* Gm^{R}	This study
R7AΔ*dct nadQCMD::pFUS2*	R7AΔ*dctABD nadQCMD::pFUS2* Gm^{R}	This study

R7AΔ*dct*Δ*nadQ* *nadB::pFUS2*	R7AΔ*dctABD nadB::pFUS2* Gm^R Δ*nadQ* markerless deletion mutant	Provided by John Sullivan
E. coli		
S17-1	*recA thi pro hsdR hasM*, [RP4-2(Tc::Mu)(Km::Tn7)]	(65)
HB101	*leuB thi xyl mtl ara* D(*gpt-proA*) *lacY hsdS recA galK supE mcrB*, Str^R.	(9)

Table 2.2: Plasmids used in this study.

Plasmid	**Characteristics**	**Source**
pLAFR1	Broad-host-range cosmid; pRK290 derivative with λ *cos* site, *oriV*, *oriT*, *IncP1*, single *Eco*RI site. Tc^R.	(31)
pJQ200SK	pACYC184-derived (p15A) suicide vector, *sacB*, Gm^R.	(73)
pRK2013	$oriV^{RK2}$, Mob^+, Tra^+, Kan^R Helper plasmid.	(25)
pIJ3200	pLAFR1 derivative containing the poly-linker of Bluescript M13, with λ *cos* site, *oriV*, *oriT*, *IncP1*, Tc^R	(59)
pFAJ1700	Broad-host range cosmid used for cloning and transfer of *fixV*; pRK290 derivative with *oriV*, *oriT*, MCS site. Tc^R.	(17)
pFUS2	Suicide vector, $oriV^{R6K}$, *oriT*, Gm^R	(3)
p637	pLAFR1 containing a 23.14 kb region of the R7A symbiosis island including *dctABD*, *nadQABC* and *bio* genes	(99)
p686	pLAFR1-based cosmid from R7A genomic library containing symbiosis island DNA; insert overlaps that in p637 and includes *nadQABC* genes, *dctA* and part of *dctB*.	J. Sullivan
p993	NotI/BamHI restriction fragment from p637 cloned into pIJ3200; contains *nadABC* and a 3-prime portion of *nadQ*	J. Sullivan
p994	BamHI restriction fragment from p637 cloned into pIJ3200; contains *nadQABC*	J. Sullivan
p1018	BamHI/XbaI restriction fragment plus XbaI PCR product with 3-prime end of *nadC* and the downstream gene, both from p637, cloned into pIJ3200; contains *nadQABC* but the XbaI PCR product was cloned in the wrong orientation, resulting in a slightly truncated *nadC* gene.	J. Sullivan
pJW5	pLAFR1-based cosmid from *M. loti* strain $CJ1Dct^+$ library, confers mutator phenotype. Tc^R.	(112)

Table 2.3: Primers used in this study.

Primer name	**Sequence**	**Use**
nadAfwdpfus	TTTAAAAAGCTTTCACTGCGTGGC TGACAT	*nadA* knockout pFUS2 vector
nadArevpfus	AAATTTGGTACCCAGCATGATCA CACGCGG	*nadA* knockout pFUS2 vector
nadBfwdpfus	TTTAAAAAGCTTGGAGCATTGTA GGGGTGG	*nadB* knockout pFUS2 vector
nadBrevpfus	AAATTTGGATCCGGCTGCGAGCT GATCCGA	*nadB* knockout pFUS2 vector
nadCfwdpfus	TTTAAAAAGCTTGTTGCCGGGCTC GACTTG	*nadC* knockout pFUS2 vector
nadCrevpfus	AAATTTGGATCCCTTGATGAGCA CGGCGTC	*nadC* knockout pFUS2 vector
nadAcmdF	AAATTTAAGCTTCGGCACAAATT GCCGAT	*nadA* cis-mero diploid pFUS2 vector
nadAcmdR	TTTAAAGGATCCGGTCTCGGCCAT AAAATG	*nadA* cis-mero diploid pFUS2 vector
msi362CMDL	TTTAAAAAGCTTCCAATCAAGTTG GCGAGA	*msi362* cismeric diploid pFUS2 vector
msi362cmdR	AAATTTGGATCCTTTGGCCTGATC CATGGT	*msi362* cismeric diploid pFUS2 vector
nadApfuscnfrm	CGGTCTTCGTCGAGGATATC	*nadA* pFUS2 knockout upstream confirmation
nadBpfuscnfrm	ACCTCAAGCCGTCGAGAACCT	*nadB* pFUS2 knockout upstream confirmation
nadCpfuscnfrm	CACGAGCGATGCGATCATTC	*nadC* pFUS2 knockout upstream confirmation
lacZ	GCTATTACGCCAGCTGGCGA	internal pFUS2 *lacZ* gene primer
willynudixFwdpfus	AAATTTAAGCTTGTAGAACTGGA C	*nadQ* pFUS2 knockout

willynudixrevpfus	TTTAAAGGATCCCGCGAGCTCTTCGTTCCA	*nadQ* pFUS2 knockout
msi362cmdpfusconfirm	AGCGCGCGAGGAGAATAGCT	*nadQ* cis-mero diploid pFUS2 vector upstream confirmation
nadAcmdpfusconfirm	CTCCGGCAGGCTCATCAACA	*nadA* cis-mero diploid pFUS2 vector upstream confirmation
msi362knockconfirm	GACCAACGATGTCAACGCCG	*nadQ* knockout pFUS2 vector upstream confirmation

Table 2.4: Antibiotics used in this study.

Antibiotic	**Abbreviation**	***E.coli*(µg/mL)**	***M. loti* (µg/mL)**
Tetracycline	Tc	15	2
Gentamicin	Gm	25	50
Rifampicin	Rif	--	100

2.6 Electroporation

2.6.1 Preparation of electrocompetent cells

For *E. coli* S17-1, cells 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 37°C with shaking at 200 rpm. Cultures were harvested at OD_{600} 0.6-0.8 by centrifugation at 4°C for 10 min at 5930 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, aliquoted into 40 µl volumes and then snap-frozen in a dry ice/ethanol bath. Cells were stored at -80°C (91).

For *Mesorhizobium*, 5-ml TY broth cultures of strains were grown to stationary phase (72 hours) with 1 ml then used to inoculate 200-ml TY broths which were incubated at 28°C with shaking. Cells were harvested at OD_{600} 0.1-0.3 by centrifugation at 4°C for 10 min at 5930 g. Pelleted cells were washed with 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 -80°C.

2.6.2 Electroporation of cells

The 40-µl aliquots of electrocompetent bacteria were thawed and mixed with 2 µl of DNA. The cells were then transferred to a chilled 1-cm gapped electroporation cuvette. Cells were transformed at 1500 V using an *E. coli* TransPorator system (BTX). One mL of LB or TY (for *E. coli* or *Mesorhizobium* respectively) was added immediately after the electroporation and transferred to a Bijou bottle. The cultures were incubated at 37°C with 200 rpm shaking for 40 min for *E. coli* or 28°C with 180 rpm shaking for 3 h for *M. loti* respectively.

2.7 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).

2.8 Frozen inocula

Relevant strains were picked from single colonies on GRDM plates and grown for 72 hours in 5-ml G/RDM broths. Broths were then dispensed into cryotubes in 900-µl aliquots with 100 µl of glycerol to yield a final concentration of 10% glycerol. Inocula were then frozen and stored at -70°C prior to use.

2.9 Dct reversion assay

One hundred µl of prepared frozen inocula were added to 5-ml TY broths and grown for 72 hours. Cells were then harvested by centrifugation at 7000 rpm from 4 ml of broth and resuspended in 4 ml of sterile H_2O. These solutions were then adjusted to reach on OD of 1.00 +/- 0.1 to yield a standard dilution. A dilution series ranging from 10^0 through to 10^{-6} was then done for each strain. Dilutions 10^0 through to 10^{-3} were then plated in duplicate onto S/RDM and incubated at 28°C for 15 to 20 days. New Dct^+ colonies were counted daily in an additive manner. Dilutions at 10^{-6} were plated onto G/RDM in triplicate, with colonies counted after 5 days in order to establish the initial c.f.u. of each strain. Any experiment-specific modifications to this basic design are covered in the relevant results section.

2.10 Catalase assay

The catalase used was bovine liver catalase in liquid form as supplied by Sigma (Catalogue number C30) at an original concentration of 34 mg/mL, with 10, 000 enzyme units per mg of protein. This was diluted to produce a final concentration of 100 enzyme units per 100 µL as

described by Flowers *et al.*(26). This solution was then filter sterilized and 100 µL was spread on S/RDM plates and the plates dried with their lids removed in a laminar flow hood.

2.11 Bacterial crosses

Bacterial crosses were generally performed through triparental spot mating, in which 25 µl of a TY culture of each strain was mixed as a drop on TY plates and allowed to dry exposed in a laminar flow hood for approximately 45 minutes and then incubated overnight at 28°C. The resulting growth was then streaked directly onto GRDM containing the appropriate antibiotics and incubated for 4 days at 28°C. Single colonies were then passage-purified a further 3 times on GRDM under the same conditions.

2.12 Screening of cosmid libraries

Two cosmid libraries of R7A constructed through partial restriction digest of genomic DNA with EcoRI or Sau3A were available. These libraries were a mixture of individual clones from 96-well micro-titre plates added to make 4 communal cultures that were incubated in TY broth. One ml of each culture was then mixed with 1 ml of R7ANS and HB101/pRK2013 respectively. The mixtures were then absorbed onto 47-mm nitrocellulose filters, which were then incubated on TY plates at 28ºC for 24 h. The 4 matings were then resuspended in TY broths and a dilution series of 1 through to 10^{-7} prepared. Each dilution was then spread in triplicate on SRDM + Rif + Tc. Suspected Dct^{+} colonies were then selected and single-colony purified three times before being used as donors in matings back into *E. coli* to recover plasmids for sequencing.

In the second library screen a similar procedure was followed except the dilution series was placed on GRDM + Rif + Tc. Single colonies were then picked at random and streaked onto SRDM plates.

2.13 DNA preparation

2.13.1 Plasmid DNA extraction

2.13.1.1 Boiling minipreps

A boiling miniprep method was used to extract plasmid DNA. Bacteria were grown in TY broths with appropriate antibiotics at 37°C with shaking for 18 h. Bacteria from 1.5 ml of culture were harvested by centrifugation with pellets then re-suspended in 350 µl HQ-STET buffer (8% [w/v] sucrose, 50 mM EDTA [pH 8], 5 mM Tris [pH 8], 5% [w/v] Triton X-100). Lysozyme, 25 µl of (10 mg/mL in 10 mM Tris [pH 8]), 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 MilliQ water.

2.13.1.2 Alkaline lysis preparations

Plasmid DNA was also extracted using alkaline lysis (24). Overnight cultures (3 ml) grown in TY broths at 37°C were harvested by centrifugation. Pelleted cells were re-suspended in 250µl Buffer P1 (QIAGEN) before being lysed by the addition of 250 µl lysing solution (0.2 M NaOH, 1% [w/v] SDS) and vortexing, followed by incubation on ice for 3 min. Neutralizing 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 µl of 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 resuspended in 50 µl of filter-sterile MilliQ water.

2.13.1.3 Plasmid and cosmid isolation using commercial kits

High quality plasmid DNA was isolated using a QIAGEN plasmid midi kit. Cosmid, plasmid and insert DNA for use in subcloning was prepared using QIAprep spin miniprep kit.

2.13.2Genomic DNA isolation

2.13.2.1Ultra-quick genomic DNA preparations

Genomic DNA of *Mesorhizobium* sp. strains was isolated by modification of the ultra-quick genomic DNA preparation method. 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 of 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 chloroform. Genomic DNA was then precipitated using isopropanol, washed in 70% ethanol and re-suspended in 50 µl of filter-sterile MilliQ water. To aid the resuspension of the DNA, tubes were incubated at 55°C for 1-2 h.

2.13.2.2 PrepMan^{TM} 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.

2.14 PCR reactions

PCR reactions were performed using the Phusion High-Fidelity PCR Kit (Finnzymes). PCR was done in 100 µl volumes consisting of 20 µl of 5x high fidelity buffer, 3 µl of dimethyl sulfoxide (DMSO), 2 µl of dNTPs, 1 µl of each primer and DNA template while the remaining volume was made up with filter-sterile MilliQ water. A Hybaid PCR Express Thermal Cycler was used to perform the PCR reactions. A typical PCR reaction involved a first cycle at 98°C for 1 min. The second cycle was at 98°C for 10 s, 57°C for 15 s and then 72°C for 20 s, the whole cycle being repeated 30 times. The third and final cycle was 98°C

10 s, 57°C 15 s and 72°C for 5 min. PCR products were column purified for sequencing using the High Pure PCR Product Purification kit from Roche Applied Science.

2.15 Restriction digests

Plasmid DNA was digested with EcoRI in the manufacturer's (Roche) recommended buffers at 37°C for 3 h. Sixty-µl restriction digest reactions were prepared composed of 20-µl DNA, 6-µl 10X buffer, 1-µl EcoRI with the remaining volume made up with MilliQ water.

2.16 Agarose gel electrophoresis

DNA was mixed with bromophenol blue tracking dye and separated on 1% agarose gels made and run with 1x Tris-acetate EDTA buffer containing 1 µg/ml ethidium bromide. Typically gels were electrophoresed for a ~1 h at 90-100 V (except for gels used for Southern blotting which were electrophoresed at 22 V overnight). Gels were then visualized and photographed under UV illumination.

2.17 DNA sequencing

Sequencing was performed at the Allan Wilson Centre Genome Service at Massey University (Palmerston North). Template and primer were pre-mixed in a total volume of 15 µl for PCR products and a total volume of 12 µl for cosmid DNA. Primers for sequencing were diluted to 3.2 pmol/15 µl. The template concentration was dependant on the size of the product and source.

2.18 Southern hybridization

2.18.1 Downward capillary blotting

Transfer of genomic DNA after electrophoresis from 1% agarose gels to Hybond-N^+ membranes (Amersham BioScience) was achieved by immersing the gels for 15 min in 0.2 M HCl, rinsing twice in dH_2O prior to submersing in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 15 min, followed by a final 15 min soak in neutralisation solution (1.5 M NaCl, 0.5 M Tris [pH 8]). The downward capillary system involved the construction of a stack of the following: a 7-cm high stack of paper towels formed the base of the stack, on top of this 4 dry and 1 wet (soaked in 20 x SSC) pieces of Whatman filter paper were placed. The membrane (pre-soaked for 5 min in dH_2O, followed by 5 min in 20 x SSC) was then placed on the stack and the treated agarose gel that had the wells cut off was placed on top of the membrane, ensuring no air bubbles formed. Finally, 3 more pieces of pre-soaked Whatman paper, 2 of which were cut with an extension that was placed in a reservoir of 20 x SSC, were placed on the top of the stack. The stack was left for ~3 h for transfer of the DNA to occur. The DNA was then fixed to the membrane by soaking the membrane in 0.4 M NaOH for 20 min. Finally the membrane was rinsed in 20 x SSC and air-dried. To confirm the transfer of DNA visually, the membrane was placed DNA-side down on a UV transilluminator and examined for fluorescence.

2.18.2 Hybridization of probe to membrane-bound DNA

Probe DNA (100 ng)and 10 ng of λ molecular marker DNA (where applicable) was labeled using the Amersham AlkPhos Direct™ labeling system as per the manufacturer's instructions and incubated for 30 min at 37°C. Membrane prehybridisation was carried out in a Hybaid bottle containing AlkPhos Direct hybridization buffer (0.125 ml/cm^2 of membrane) that was incubated at 55°C in a rotating Hybaid hybridization oven for 15 to 20 min. Labeled probe DNA was then mixed with hybridization buffer and added to bottles which were left to rotate at 55°C for 18 h. Following hybridisation, membranes were washed firstly in 2 X SSC/0.1% SDS for 10 min at 55°C, then 1 X SSC/0.1% SDS for 20 min at 55° C, followed by a final 20 min wash in 0.1 X SSC/0.1% SDS at 55°C. Three ml of detection reagent was then applied to the membranes, which were left for 5 min, then drained and sealed in plastic and exposed to

X-Ray film (Cronex, Dupont) for 4-72 h in the presence on Quantra-rapid or Lightening-plus intensifying screens.

2.19 β-galactosidase assays

Cultures were grown to mid-exponential phase and harvested, with the pellet resuspended in an equal amount of Z buffer and the absorbance measured at OD_{660}. In the case of cultures that were grown to stationary phase, the resuspended solution was diluted to an OD_{660} of ~ 0.8. Three aliquots of each culture (100, 200 and 400 µl) were dispensed and brought to a final volume of 1 ml by the addition of assay buffer. Forty µL of $CHCl_3$ and 20 µl of 0.1% SDS were added and the cells permeabilised for 5 s via vortexing followed by standing for 5 minutes. Reactions were begun with addition of 200 µl of 25 mg/ml ONPG. When the reaction reached a yellow colouration (~OD_{420} 0.1-0.4) it was stopped via addition of 500 µl of 1M sodium carbonate. Cells were removed form suspension by centrifugation and the absorbance was measured at OD_{420}. The enzymatic activity in Miller units was calculated using the following formula:

1000 x (OD_{420}/t x V x OD_{660}), where t is the time (min) the reaction took to proceed and V is the volume (ml) of cell suspension in the assay.

2.20 Computer analysis of DNA sequence data

DNA sequences were viewed and edited using the computer program SeqEd V1.0.3 (Applied Biosystems) and assembled into contigs using SeqMan (DNAStar). National Centre for Biotechnology Information (NCBI) databases were searched for similar nucleotide or amino acid sequences using Blast N, X and P.

2.21 UV exposure

Five-ml starter cultures were grown in TY for 72 h. Fifty-ml TY broths were then inoculated with 100 µl of starter culture and grown for a further 24 h. These were then spun at 7000 rpm

for 15 min, the supernatant discarded and the pellet resuspended in 30-mL 0.9% NaCl solution. One-ml aliquots of each strain were poured onto glass plates and exposed to a UV lamp (240 nm) for 4, 9 and 12 s (individual aliquots for each time interval). Cultures of each strain at each time interval for each strain were diluted 10^{-2}, 10^{-4} and 10^{-6} (in saline) with 100-μL aliquots spread on duplicate GRDM plates. A second dilution series was also run for each strain not exposed to UV (time 0). Plates were then incubated at 28°C in the dark for 5 days. All UV exposures and subsequent plating were conducted in a darkroom

3. Results

3.1 R7AΔ*dct* phenotype

The *M. loti* strain R7A contains two copies of the *dct* locus, a functional copy on the symbiosis island and an apparently non-functional 'cryptic' chromosomal copy. This means that R7A is able to utilise succinate as a sole carbon source (Dct^+) but when the island *dct* locus is removed, such as in the R7AΔ*dct* mutant strain, it becomes unable to utilise succinate (Dct^-). However, after an extended period of incubation (~5 days plus) on solid medium containing succinate as the sole carbon source, colonies that are Dct^+ appear in large numbers, and continue to appear for ten days or more (112) (Figure 3.1). For the lower dilutions (10^0 to 10^{-3}), 300-450 Dct^+ colonies typically appear from the surrounding Dct^- cell lawn. The majority of Dct^+ colonies arise in the first ~10 days after plating, with colony appearance tapering off significantly but colonies still appearing up until at least 20 days after plating. At these dilutions the total number of Dct^+ colonies that appear is similar i.e. does not show a 10 fold decrease in the number of Dct^+ colonies that would be expected from 10 fold dilutions (Fig 3.1a). This is likely due to the lower dilutions exhausting succinate in the medium locally preventing nearby colonies from arising. This is reflected by the delay in peak colony appearances which roughly follows the dilution series, and that the 'peaks' rise and fall far more sharply at lower dilutions (Figure 3.1b).

In contrast to R7AΔ*dct*, R7ANS, which possesses the chromosomal *dct* locus but not the symbiosis island, does not produce Dct^+ colonies (Figure 3.2). This strongly suggested that a factor located on the island was responsible for this phenotype. The aim of this work was to identify and characterise that factor.

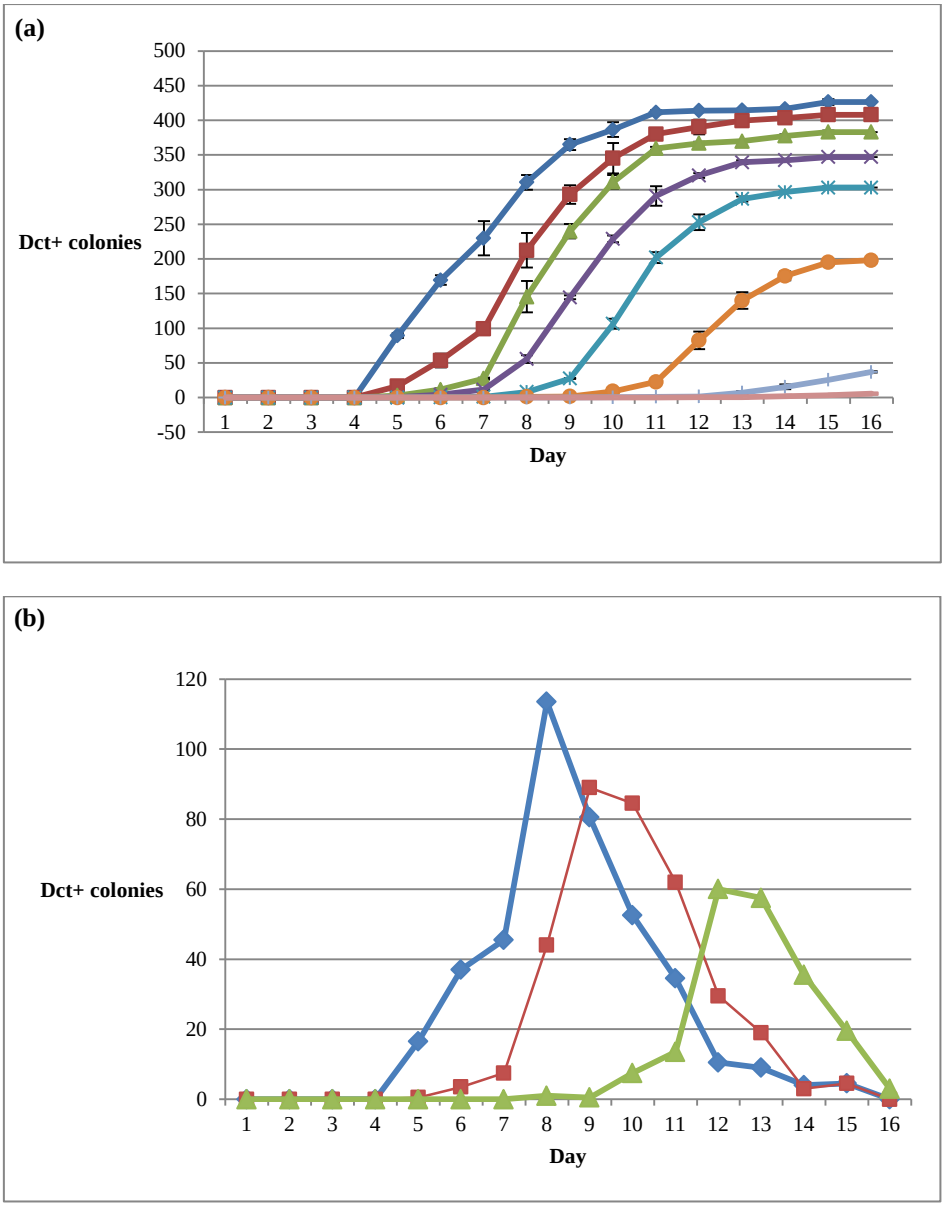


Figure 3.1: (a) Graph showing appearance over time of Dct$^+$ colonies after plating various dilutions of a TY culture of strain R7AΔ*dct* . Dark blue diamonds represent plating of 0.1 ml of an undiluted culture, maroon squares a 10^{-1} dilution, green triangles a 10^{-2} dilution, purple tick and crosses a 10^{-3} dilution, teal asterisks a 10^{-4} dilution, orange circles a 10^{-5} dilution, light blue crosses a 10^{-6} dilution and pink dashes a 10^{-7} dilution. The TY culture plated contained 7 x 10^8 colony-forming units per ml, as estimated from total number of colonies (including Dct$^-$ microcolonies) on plates inoculated with the 10^{-7} dilution. (b) Average number of Dct$^+$ colonies appearing each day at three selected dilutions of R7AΔ*dct*. Blue diamonds represents 10^{-1} dilution of a the culture, maroon squares a 10^{-3} dilution and green triangles a 10^{-5} dilution.

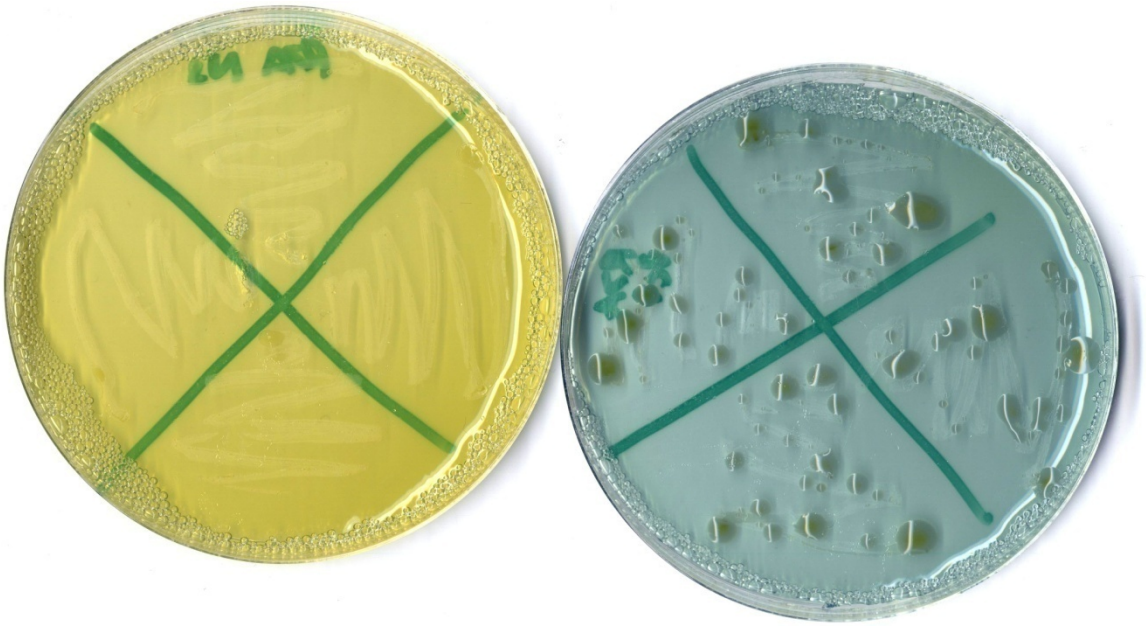


Figure 3.2: Two plates showing a comparison of the phenotype between R7ANS andR7AΔ*dct*. Each quadrant of the plate was streaked from a separate single colony off a GRDM plate. The plate on the left was streaked with strain R7ANS and shows very little in the way of growth, with only very small background microcolonies most likely utilizing impurities present in the medium. The plate on the right represents R7AΔ*dct* which is able to form large succinate-utilizing colonies that began appearing sporadically after ~5 days of growth. Colour difference is due to change in pH caused by the utilization of succinate.

3.2 Identification of cosmid p686 that conferred the mutator phenotype.

As it was apparent that a factor on the symbiosis island determined the R7A mutator phenotype, selection screens were carried out using two preexisting cosmid libraries of strain R7A, one prepared with genomic DNA partially digested with EcoRI and the other with DNA partially digested with Sau3A. The screens were carried out by mating the library clones into R7ANS, selecting on GRDM containing tetracycline and picking and streaking random colonies (~720 per screen) directly onto succinate plates. As the R7A genome is approximately 8 Mb in size and each cosmid contains approximately 25 kb of DNA, then each screen gave approximately two fold coverage of the island, with the total number of clones examined across all four screens offering a 99.99% chance of locating the clone of interest (though it was only a 89% probability for each individual screen). In total, 5 streaks at first appeared promising, however, after colony purification and sequencing of the cosmid ends, it was revealed that they all corresponded to regions that contained the island *dct* genes and were thus deemed false positives. Each library was screened a further three times, producing the similar results.

Due to the failure of the cosmid library screens, it was decided to identify and test cosmids that mapped to the symbiosis island. Six overlapping cosmid clones from the right hand end of the symbiosis island had been identified as part of the project to sequence the symbiosis island (102) and these were tested first. Of the six cosmid clones, p637 contained the full island *dct* locus and were therefore used as a positive control for unrestricted growth on SRDM. One other of the six clones, cosmid p686, showed growth that appeared analogous to that of R7AΔ*dct*, while the other four (excluding the p637 positive control) showed a phenotype similar to R7ANS (Figure 3.3).

3.2.2 Island *dct* locus remnants

On examination of the sequence map of the p686 cosmid, it was determined that it contained a partial fragment of the island *dct* locus, consisting of a full copy of *dctA* and a truncated (and thus likely nonfunctional) portion of *dctB* (Figure 3.4). Because it was possible that these remnants of the locus were interacting with the chromosomal copy (or acting independently) to confer the phenotype, there was a need to establish what parts of the island

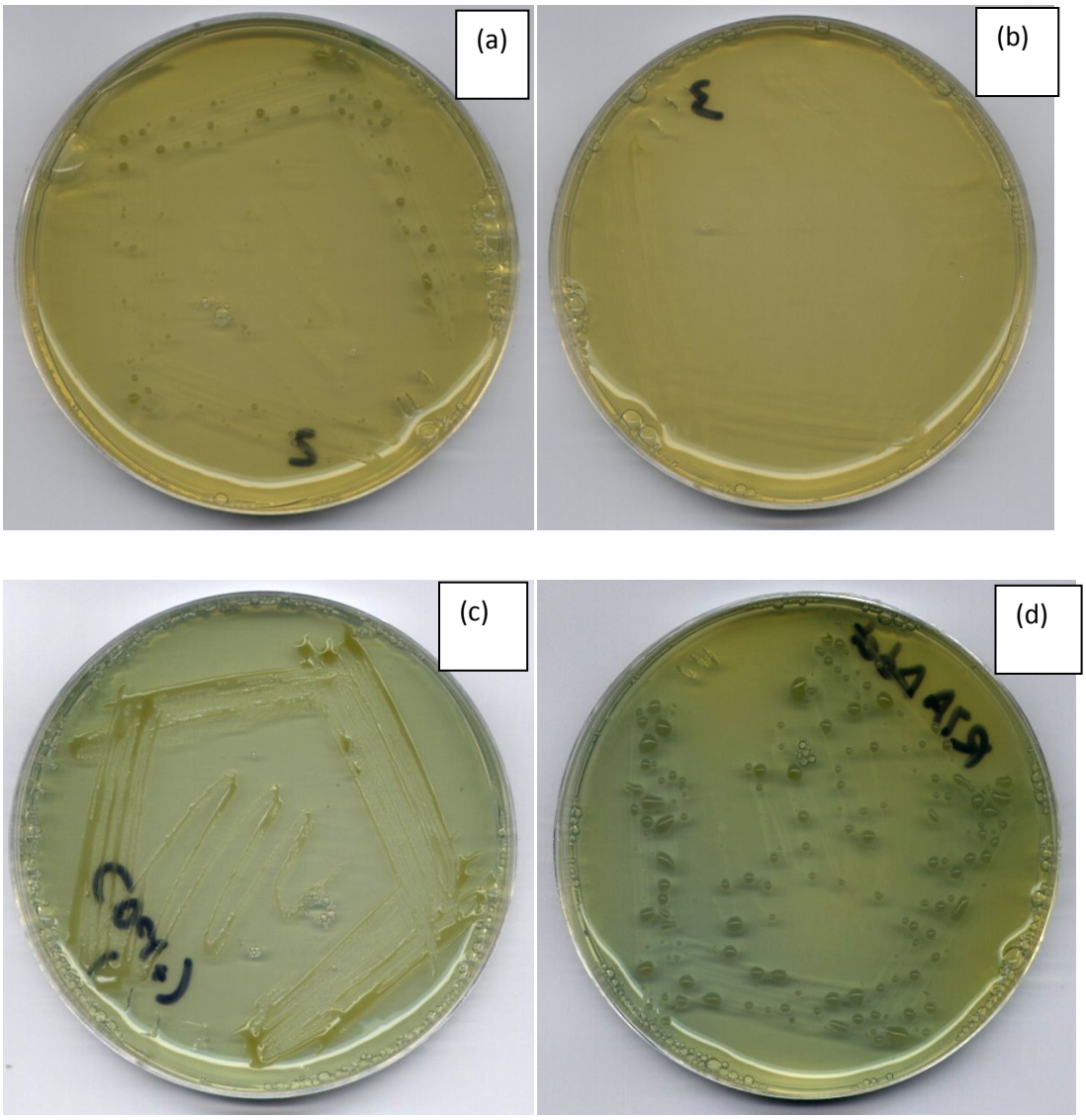


Figure 3.3*:* (a)R7ANS/p686; (b)R7ANS/p772; (c)R7ANS/p637; (d)R7AΔ*dct*. Both R7ANS/p686 and R7AΔ*dct* showed sporadic appearance of large Dct^{+} colonies, while R7ANS /p772 showed no growth and R7ANS/p637 showed uniform growth.

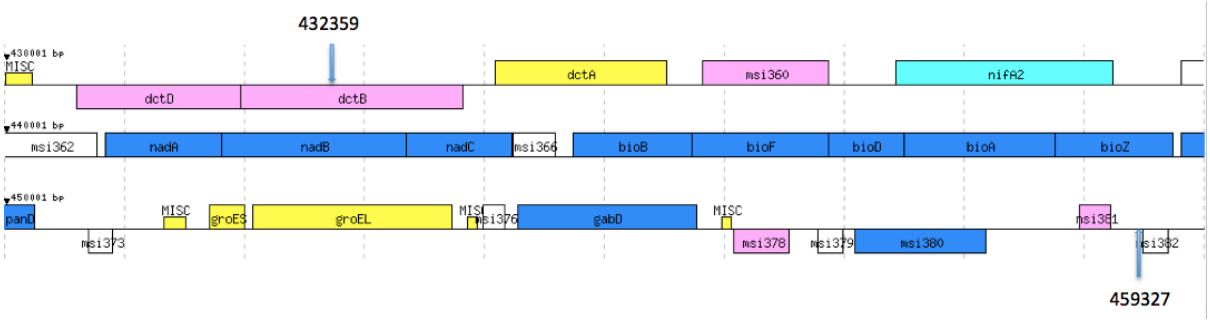


Figure 3.4: Map of the symbiosis island region encompassed by p686 with position and coordinates of the ends of the insert shown.

dct locus were essential for a Dct^+ phenotype. Four preexisting strains with deletions in the symbiosis island *dct* locus were used: R7A Δ*dctA*, Δ*dctB*Δ*dctD* and Δ*dctAB* (112). The mutants were spread on SRDM containing tetracycline and allowed to grow for 4 days. R7A was used as a positive control.

Of the strains plated, R7AΔ*dctB* and R7AΔ*dctAB* showed no growth after 4 days. The Δ*dctA* mutant showed uniform growth similar to the R7A control while Δ*dctD* showed partial growth (Fig. 3.5). This indicates that the island *dctB* gene is essential for growth on succinate medium, while the remaining two symbiosis island genes are not essential. It is possible that the chromosomal *dctA* and *dctD* genes can complement the symbiosis island *dctA* and *dctD* mutants.

3.3 *nad* locus

Concurrent to my work with the R7A-based phenotype, work had been carried out by others using the nonsymbiotic *M. loti* strain CJ1, which also displays a mutator phenotype which was determined to be conferred by an indigenously occurring plasmid. A cosmid clone that contained the region of the plasmid that conferred the phenotype had been identified and labelled pJW5. The insert in pJW5 had been sequenced (GenBank Accession number EF618553.1). As both pJW5 and p686 were able to induce the mutator phenotype in R7ANS, comparing what loci they had in common was a logical step. The comparison showed that the two plasmids shared two loci, *fixV* and *nadQABC*. A clone containing *fixV* cloned in the broad-host-range vector pFAJ1700 (17) was available in the lab culture collection. This plasmid was electroporated into *E. coli* S17, spot mated into R7ANS and the mating mixture streaked onto SRDM. The transconjugants only formed microcolonies after a prolonged incubation, thus ruling out *fixV* as causing the mutator phenotype.

Following this, three restriction fragment subclones of the *nadQABC* locus were mated intoR7ANS: p1018, p994 and p993. The three subclones were constructed in previous work (99) and all contained the *nadABC* locus. However, the subclone p1018 also contains the ORF upstream of *nadABC* that encodes a putative Nudix hydrolase-like regulator (ORF1 in Figure 3.6, now named *nadQ*) and a ~50bp intergenic sequence between this and *nadABC*. p993 contains *nadABC* and a small hypothetical downstream ORF (ORF2) with no assigned

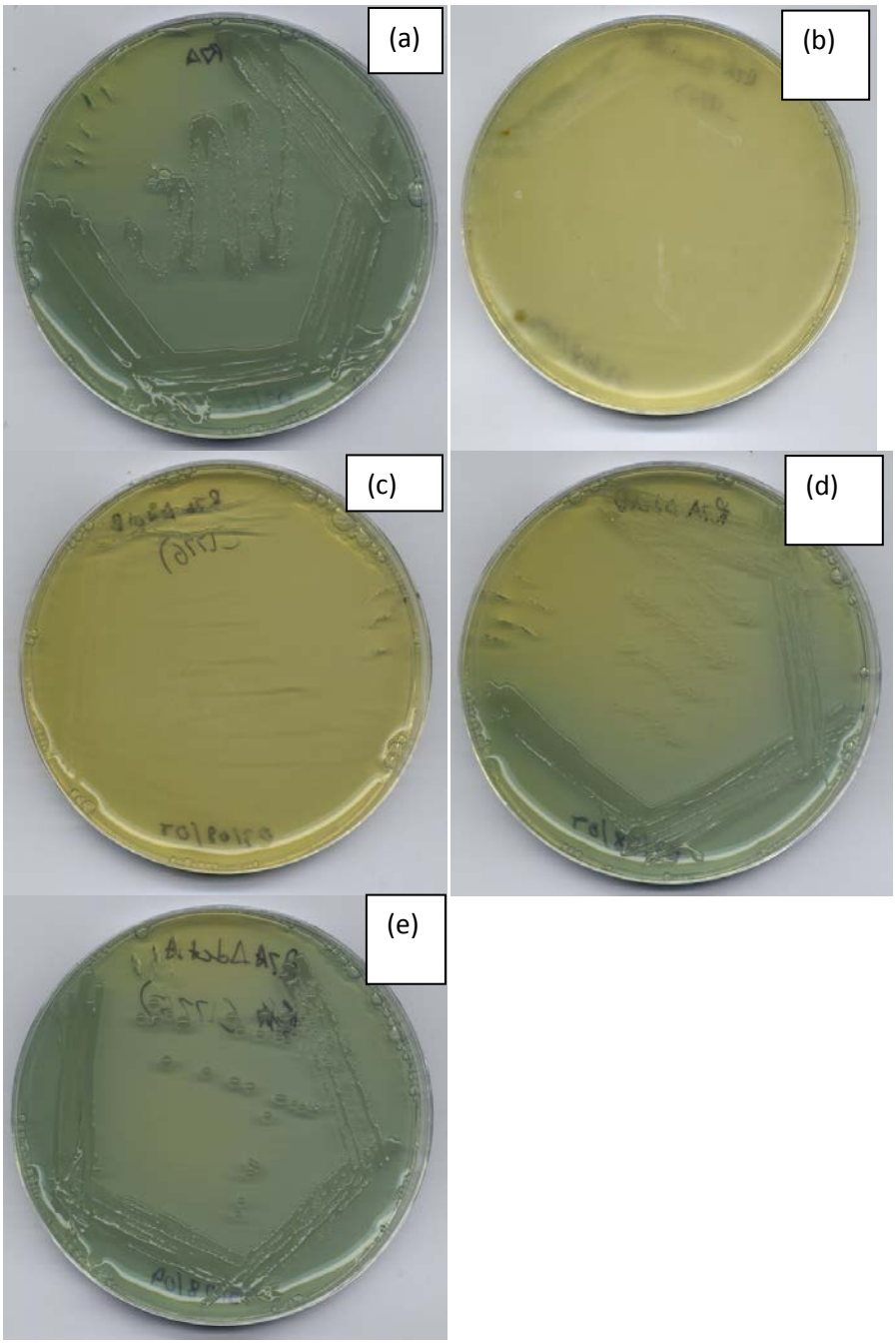


Figure 3.5: Growth of wild-type and symbiosis island mutant strains on SRDM. (a) R7A control; (b) R7AΔ*dctAB*; (c) R7AΔ*dctB*; (d) R7AΔ*dctD*; (e) R7AΔ*dct*.

function, but does not encompass *nadQ*. The subclone p994 contains the entire region covered by the other two subclones (Figure 3.6). Two of the subclones, p1018 and p994, conferred the mutator phenotype in R7ANS (Figure 3.7). This suggested that the entire *nadQABC* locus was necessary and responsible for the mutator phenotype.

3.4 Nicotinic acid

The *nad* locus identified above consists of four genes, *nadQ,* a putative nudix hydrolase-like repressor and *nadA*, *nadB* and *nadC*, which encode the *de novo* biosynthetic pathway for nicotinic acid mononucleotide (NaMN), the first part of the pathway to produce nicotinamide adenine dinucleotide (NAD). As this locus appeared essential for the mutator phenotype, it was hypothesized that the up-regulation under stress of this locus, and thus an increase in the intracellular concentration of its end-product NaMN, could be behind the phenotype. Accordingly, it seemed possible that high exogenous levels of nicotinic acid, which is utilized by the separate NAD salvage pathway, of which the end product is also NaMN, might confer the phenotype in nonsymbiotic strains and also affect the mutation frequency in strains that exhibit the phenotype.

To test this, a plating experiment was set up using R7ANS strains containing p1018 (island *nad* locus), pJW5 (region responsible for mutator phenotype in CJ1) and pLAFR1 (plasmid vector, negative control). These strains were then grown on media containing four different concentrations of nicotinic acid: 0µg/ml, 1µg/ml (concentration present in normal media preparation), 50 µg/ml and 100 µg/ml. Results showed that the mutator phenotype was indeed affected by the concentration of nicotinic acid in the medium in all three strains, though the effect depended on the strain involved (Figure 3.8). In the R7ANS/pLAFR1 control strain, which is a nicotinic acid auxotroph, high concentrations of nicotinic acid positively affected the mutator phenotype. When the concentration of nicotinic acid was either 0 or 1µg/ml the number of Dct^+ colonies appearing was virtually zero, with at most a few Dct^+ colonies appearing in the entire set of plates. However, with the addition of higher concentrations of nicotinic acid to the medium, a clear mutator phenotype appeared. Both 50 and 100µg/ml concentrations showed a massive increase in the number of Dct^+ colonies, though the total number of colonies was roughly half those seen in R7AΔ*dct*.

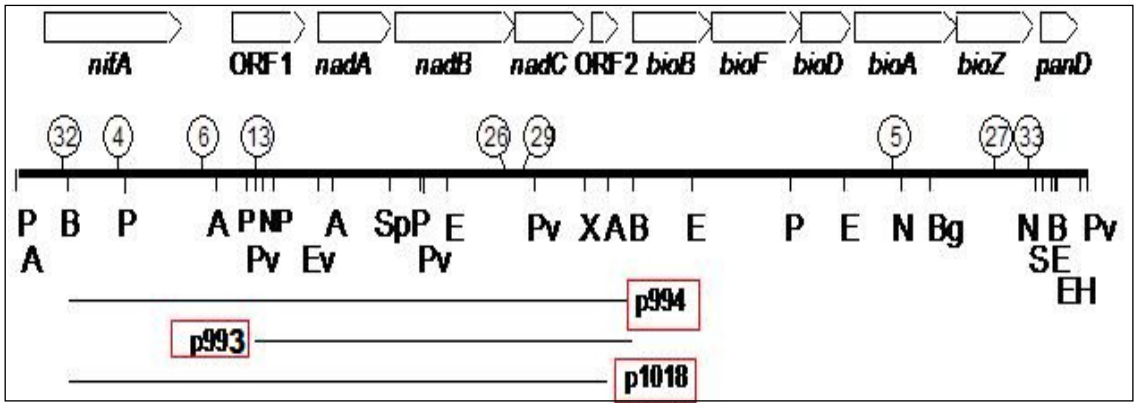


Figure 3.6*:* Map of *nadQABC* locus and surrounding genes showing restriction fragment locations and sizes. Circled number represent Tn5 insertion site while the letters represent the various restriction enzyme sites. The labeled lines at the bottom represent the relevant restriction fragments and their place in regards to the loci of concern.

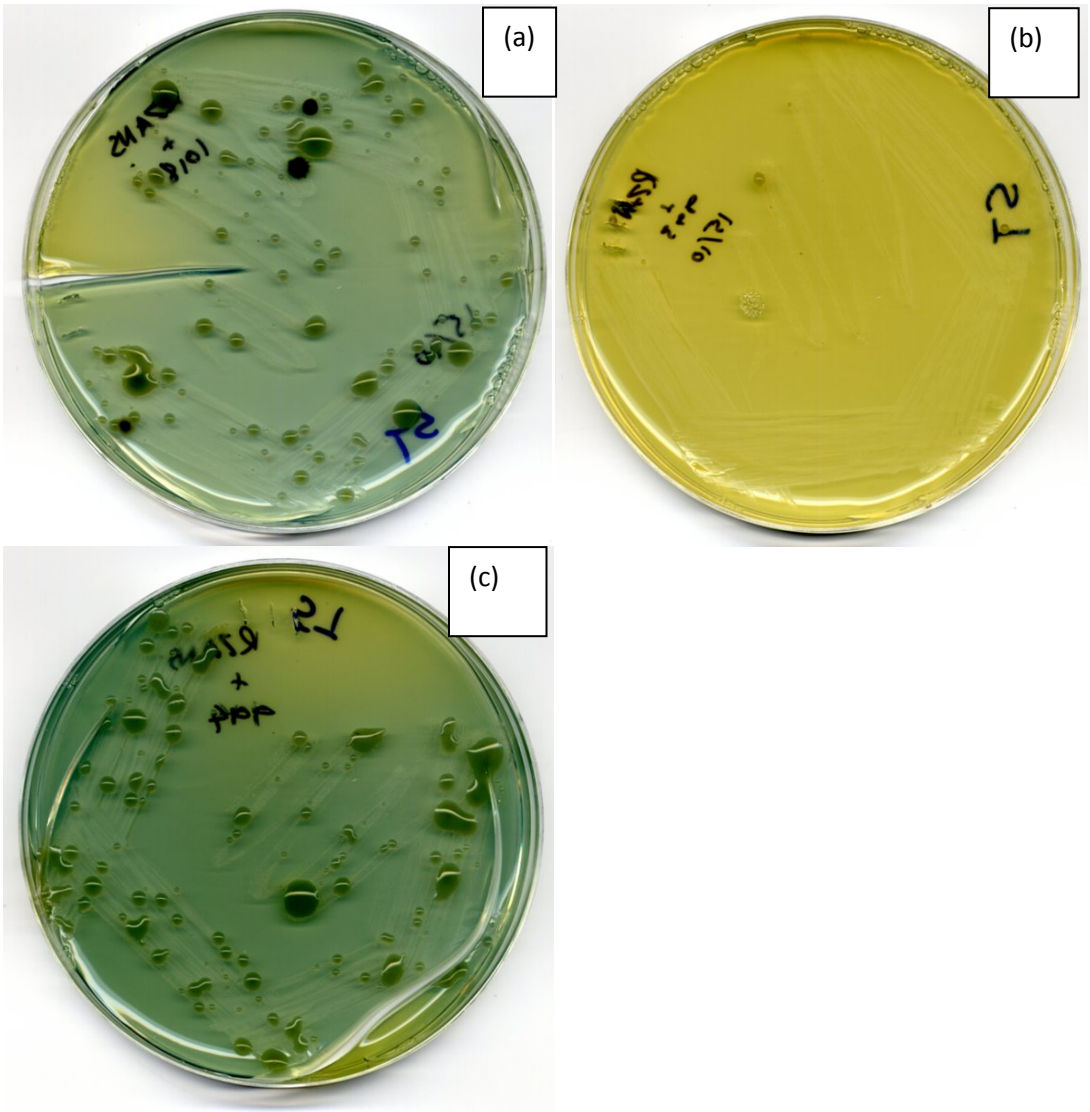
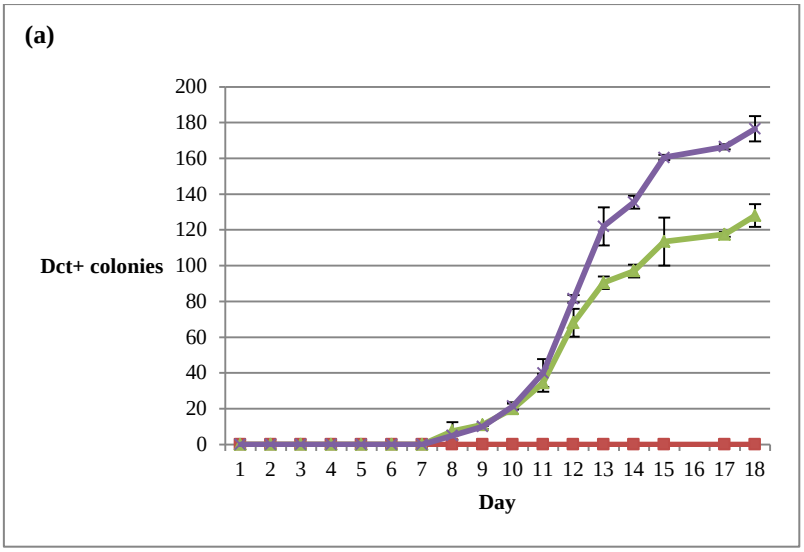
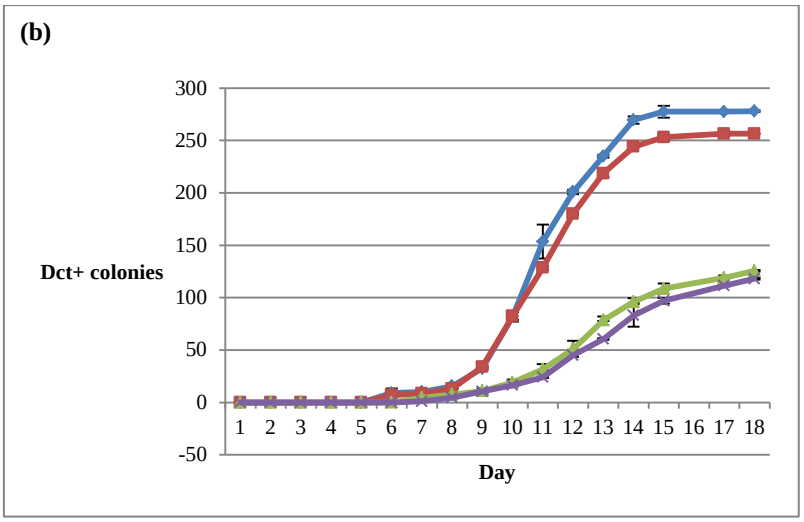


Figure 3.7: Growth on SRDM plates. (a) R7ANS/p1018; (b) R7ANS/p993; (c) R7ANS/p994.





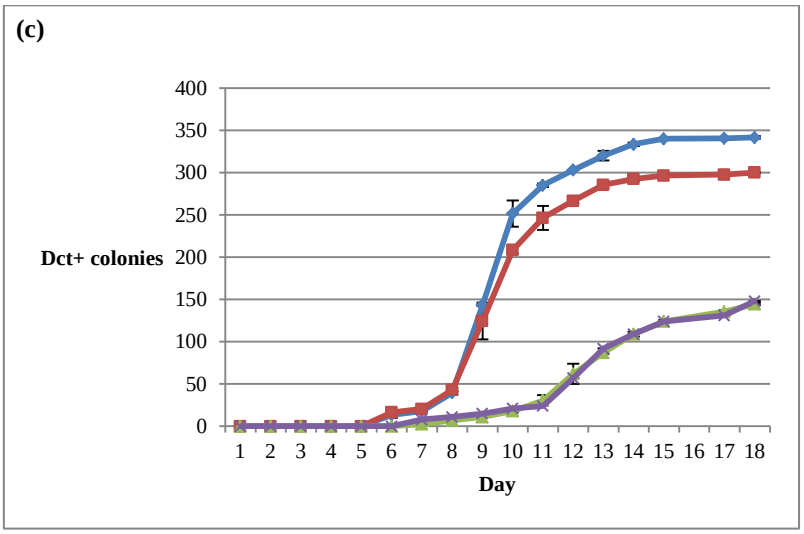


Figure 3.8: (a) Appearance over time of Dct^+ colonies on S/RDM plates containing different concentrations of nicotinic acid and spread with a 10^{-1} dilution of R7ANS pLAFR1. (b)

Appearance over time of Dct^+ colonies on SRDM plates containing different concentrations of nicotinic acid and spread with a 10^{-1} dilution of R7ANS/p1018. (c) Appearance over time of Dct^+ colonies on SRDM plates containing different concentrations of nicotinic acid and innoculated with a 10^{-1} dilution of R7ANS/pJW5. Blue diamonds represent 0 µg/ml nicotinic acid (not present in graph (a), as R7ANS is a nicotinic acid auxotroph), maroon squares represent 1 µg/ml, green triangles represent 50 µg/ml and purple ticks and crosses represent 100 µg/ml.

Furthermore, the 100 µg/ml plates showed a roughly 50% increase in colony number in comparison to plates containing 50 µg/ml, indicating that higher levels of nicotinic acid yielded higher numbers of mutant colonies.

However, the observations were reversed in both the strains that contained a functional *nadQABC* locus. R7ANS strains containing p1018 and pJW5 showed the highest number of Dct^+ colonies when no nicotinic acid was present in the medium. Numbers of Dct^+ colonies remained high at 1 µg/ml nicotinic acid, at about ~90% of those that appeared at 0 µg/ml. This small reduction was consistent across both strains and is thus likely a true reduction rather than chance variation. The two higher concentrations of nicotinic acid showed a substantial reduction in Dct^+ colonies in both strains, with the strains developing only 40-45% of the Dct^+ colonies compared to those that appeared in the absence of nicotinic acid. There was virtually no difference in the total number of Dct^+ colonies between the two higher concentrations (Figure 3.9 c and d). These results suggest that in strains possessing a functional *nadQABC* locus, the addition of exogenous nicotinic acid repressed the mutator phenotype, with the magnitude of the repression dependent on the concentration of nicotinic acid up to an as yet unknown concentration where maximum repression occurred.

At 50 µg/ml nicotinic acid, all three strains produced roughly the same number of Dct^+ colonies which was about 50% of the maximum number produced by strains with an intact *nad* locus. This suggests that the addition of large amounts of exogenous nicotinic acid was not sufficient to replicate the mutator phenotype to the levels seen when the *nadQABC* locus was present.

Lastly, there was also an apparent difference between the strains containing pJW5 and p1018, with pJW5 consistently producing a greater number of colonies, though the overall trends in relation the concentrations of nicotinic acid present were similar between the two strains (Figure 3.9).

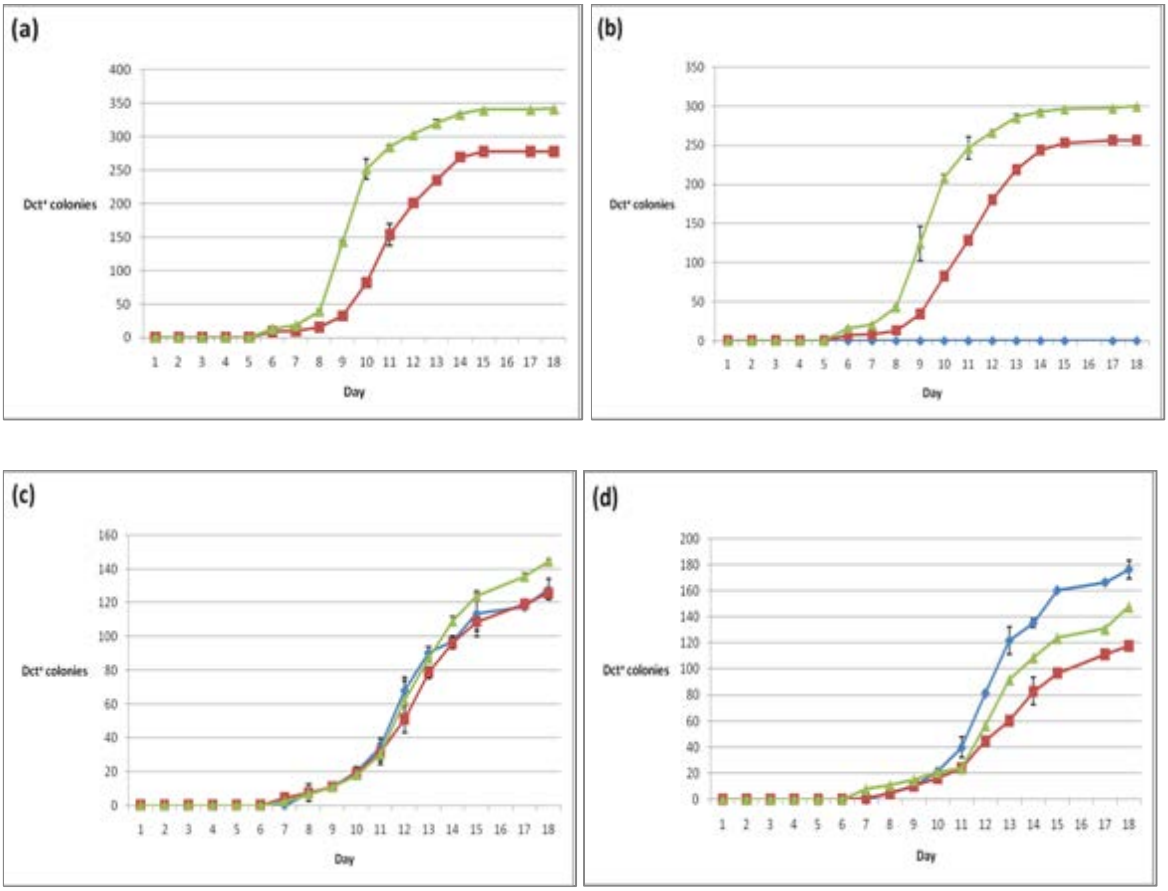


Figure 3.9: Comparison of strains. (a) Appearance over time of Dct^+ colonies in all strains when grown on SRDM medium containing a nicotinic acid concentration of 0 µg/ml. (b) Appearance over time of Dct^+ colonies in all strains when grown on SRDM medium containing a nicotinic acid concentration of 1 µg/ml. (c) Appearance over time of Dct^+ colonies in all strains when grown on SRDM medium containing a nicotinic acid concentration of 50 µg/ml. (d) Appearance over time of Dct^+ colonies in all strains when grown on SRDM media containing a nicotinic acid concentration of 100 µg/ml. Blue diamonds represent R7ANS/pLAFR1, maroon squares represent R7ANS/p1018 and green triangles represent R7ANS/pJW5.

3.5 *nad* locus mutants:

3.5.1 Construction of *nad* locus mutants

To further examine the involvement of the *nadQABC* locus in the mutator phenotype, a number of insertional duplication mutations (IDM) were constructed in the various genes of the locus. All IDM mutants were constructed using the pFUS2 vector which contains a gentamicin resistance gene and a multiple cloning site directly flanking a transcriptional fusion between fragments of *groES* and *groEL* heat shock genes and a promoterless *lacZ* gene (3). Internal fragments of the regions of interest ~ 400 bp long are cloned into the pFUS2 vector in such a way that they are in-frame with the *groELS* fragment and create a functional transcriptional fusion with the *lacZ* gene present on the vector. The vector with cloned insert is then able to recombine with the homologous genomic DNA resulting in the insertion of the vector flanked by a duplication of the cloned sequence as shown in Figure 3.10. IDM mutants were constructed in *nadQ*, *nadA*, *nadB* and *nadC*. Primers were designed to amplify and attach restriction sites to sequences for all four genes that were located in the middle portion of the gene, hence this is where the vector inserted(Figure 3.11). As this results in the insertion of a large vector into the middle of the gene, it is rendered non-functional. However, as the cloned DNA was designed to insert in-frame with the 3-prime end of a *groES* gene on the vector that is immediately followed by a hybrid gene comprising the 5-prime end of *groEL* fused to *lacZ*, this allows for cotranscription of the vector *lacZ* gene, which allowed for assessment of the levels of transcription of the target genes through β-galactosidase activity. In addition to these, cis-mero diploid (CMD) mutants were also created for both *nadQ* and *nadA*. These were created in-frame like the IDMs and thus could measure gene expression from the respective gene promoters, but did not deactivate gene activity as the genes in question were still functionally intact. All mutants were confirmed via Southern hybridization (Figures 3.12 to 3.16). Restriction fragments between 2 and 4kb (plus vector) were made from genomic DNA and probed with both the labelled original PCR product and with labelled pFUS2 vector. The resulting size of the band is based on the size of the region in the restriction fragment flanking the clone plus the duplicated cloned region and the vector itself, as summarized in Table 3.1.

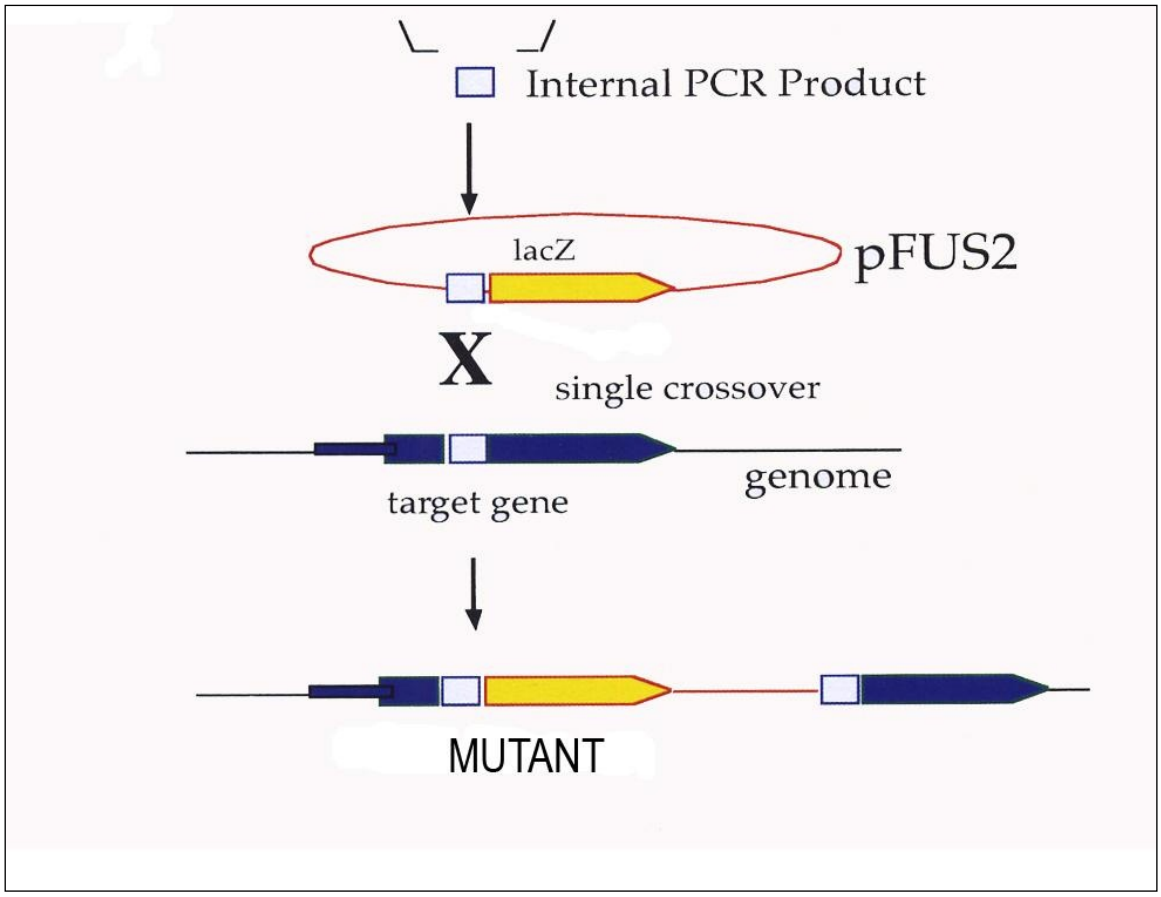


Figure 3.10: Schematic of construction of mutants using the pFUS2 vector. (Figure provided courtesy of Dr J. Sullivan).

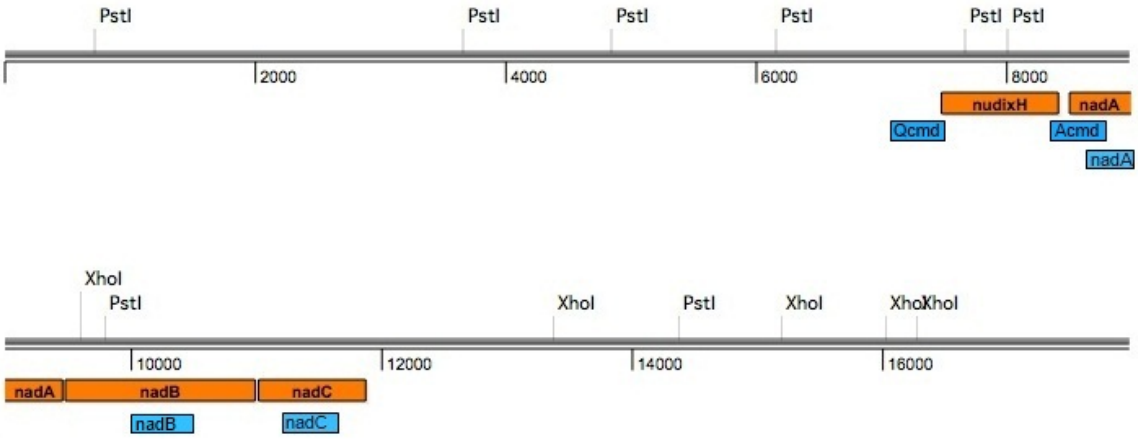


Figure 3.11: Map of the *nadQABC* locus and surrounding area showing the approximate size and location of cloned fragments used to construct mutants as well as PstI and XhoI restriction sites.

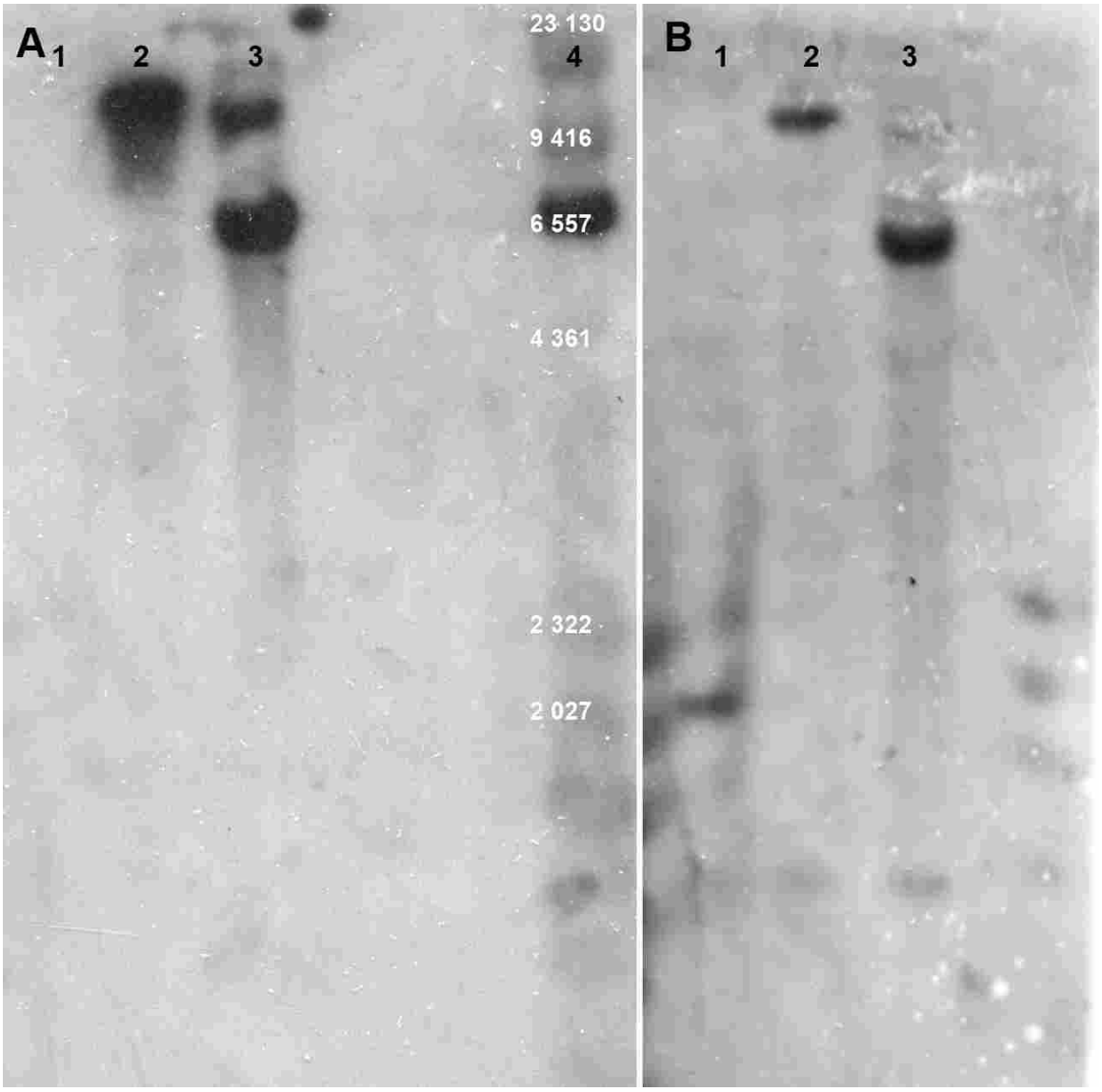


Figure 3.12: Southern hybridizations of R7AΔ*dct nadA*::pFUS2. (A) The gel probed with labelled pFUS2 vector and a small amount of labelled lambda DNA. Lane 1, which appears empty, contains R7AΔ*dct* and does not hybridize to pFUS2. Lanes 2 and 3 contain R7AΔ*dct nadA*::pFUS2 extracts, while lane 4 shows the molecular weight marker. (B) The same gel, but this time probed with labelled PCR products that were used to create the mutants. Culture represented in lane 2 was selected.

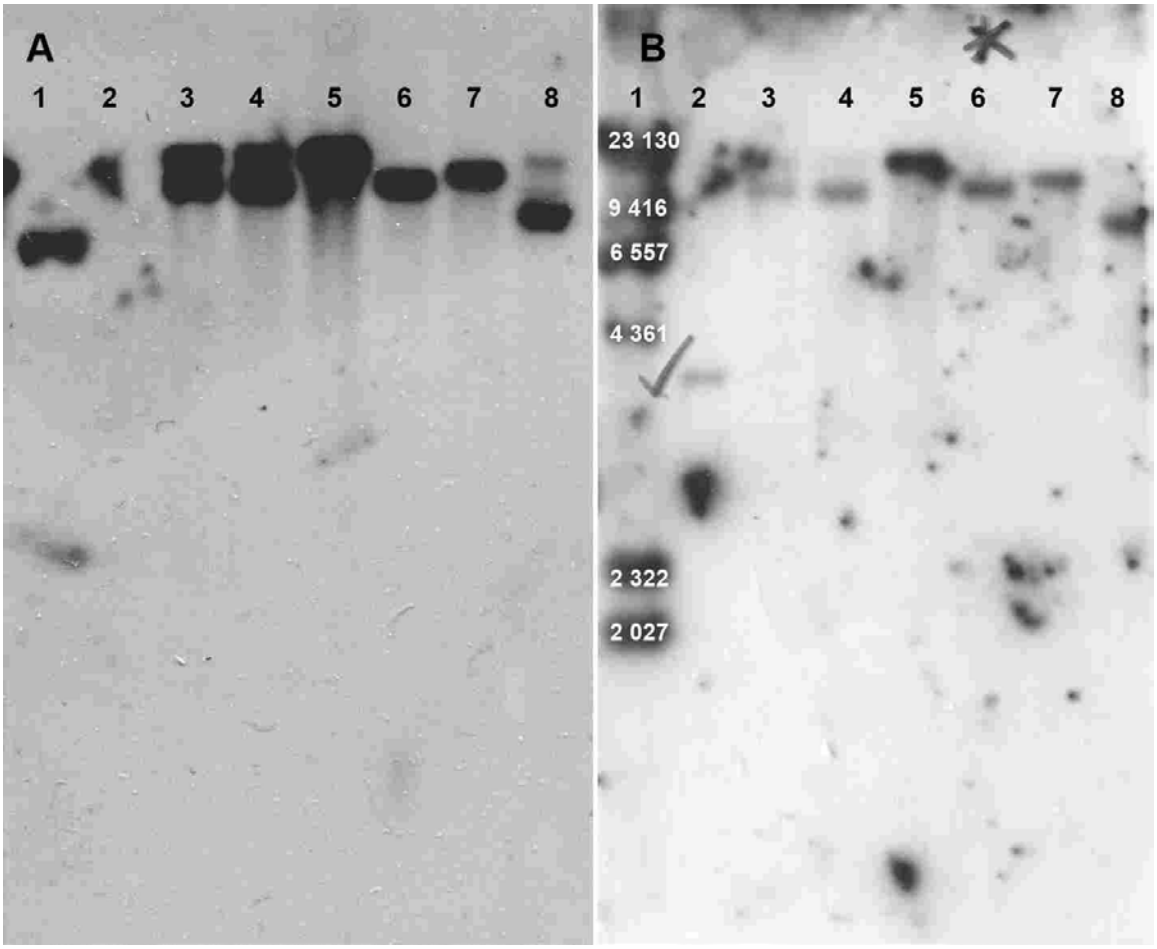


Figure 3.13: Southern hybridizations of R7AΔ*dct nadB*::pFUS2. (A) The gel probed with labelled pFUS2 vector. Lane 1 contains the molecular weight marker, in which the pFUS2 probe appears to bind to one of the molecular weight bands. Lane 2, which appears empty, contains R7AΔ*dct* and does not hybridize pFUS2. Lanes 3-8 contain R7AΔ*dct nadB*::pFUS2 extracts, which show single (6 and 7), double (5) and mixed (3, 4 and 8) insertions. (B) The same gel, but this time probed with labelled PCR products that were used to create the mutants and a small amount of labelled lambda DNA. Culture represented by lane 6 was selected.

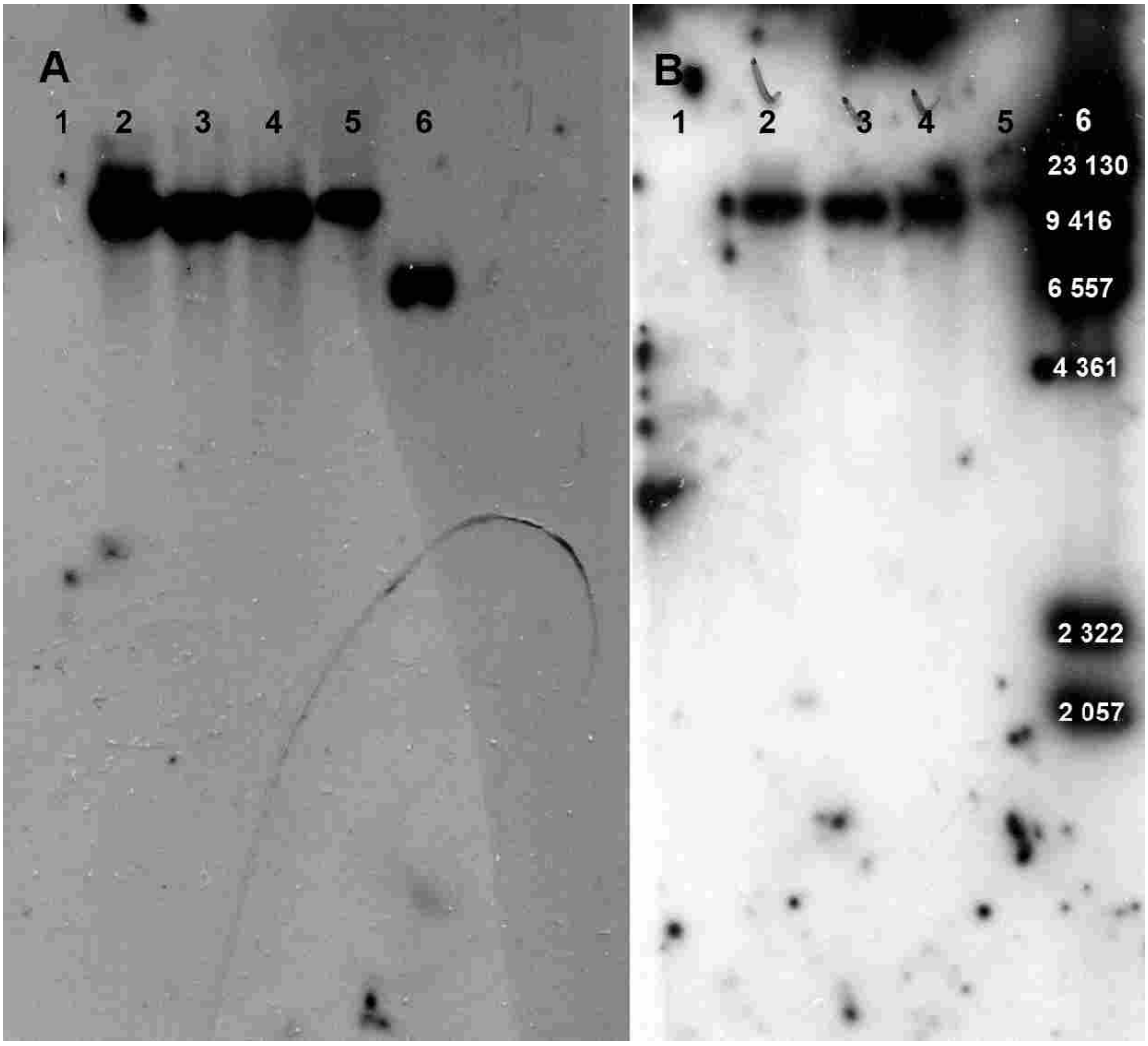


Figure 3.14: Southern hybridizations of R7AΔ*dct nadC*::pFUS2. (A) The gel probed with labelled pFUS2 vector. Lane 1, which appears empty, contains R7AΔ*dct* and does not hybridize pFUS2. Lanes 2-5 R7AΔ*dct nadC*::pFUS2 extracts, which all show single insertions. Lane 6 contains molecular marker. (B) The same gel, but this time probed with labelled PCR products that were used to create the mutants and a small amount of labelled lambda DNA. Culture represented by lane 3 was selected.

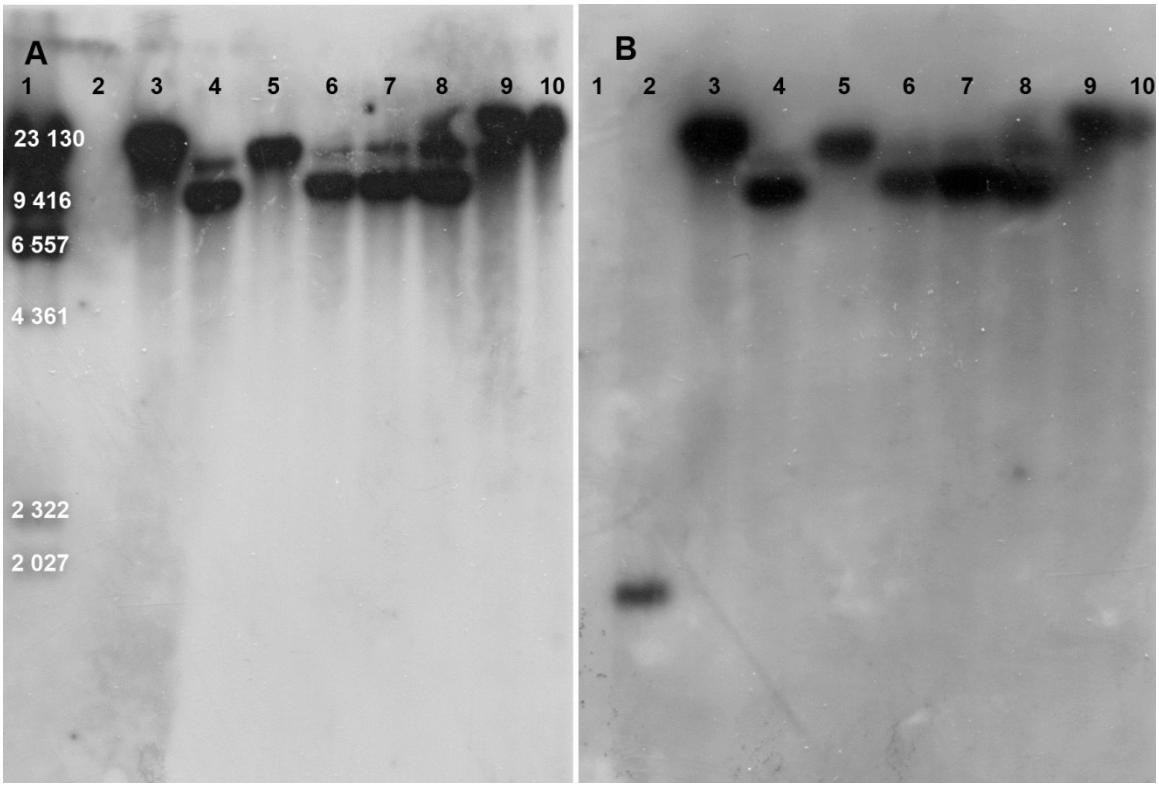


Figure 3.15: Southern hybridizations of R7AΔ*dct nadA*CMD. (A) The gel probed with labelled pFUS2 vector and a small amount of lambda. Lane 1 contains molecular weight marker with bands length labelled in white. Lane 2containsR7AΔ*dct* and does not hybridize pFUS2. Lanes 3-10 contain R7AΔ*dct nadA*CMD extracts, which show both single (6), double (3, 5, 9 and 10) or mixed (4, 7 and 8) insertions. (B) The same gel, but this time probed with labelled PCR products that were used to create the mutants and with the marker lane omitted. Culture represent by lane 6 was selected.

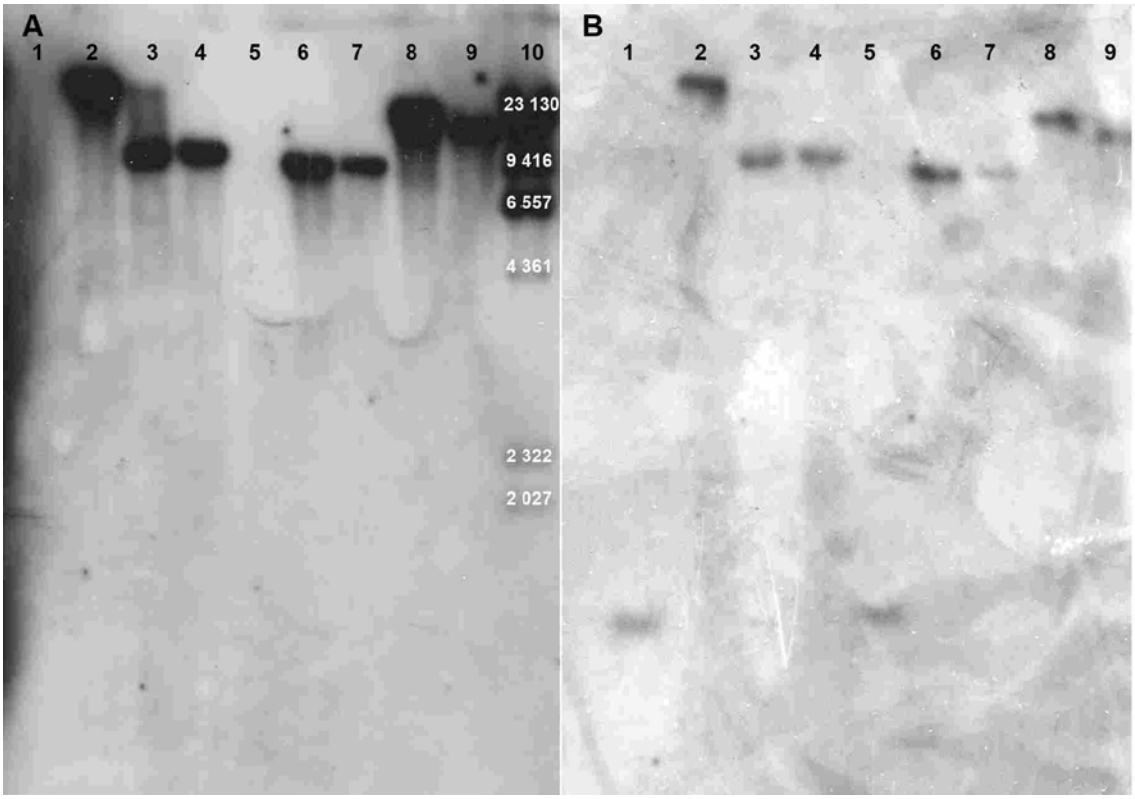


Figure 3.16: Southern hybridizations of R7AΔ*dct nadQ*CMD. (A) The gel probed with labelled pFUS2 vector and a small amount of labelled lambda DNA. Lane 1, which appears empty, contains R7AΔ*dct* and does not hybridize pFUS2. Lanes 2-9 contain R7AΔ*dct nadQ*CMD extracts, which show single (2, 4, 6 and 7) and double (2, 8 and 9) insertions, as well as one extract (5), which showed no insertion. Lane 10 contains the molecular weight marker. (B) The same gel, but this time probed with labelled PCR products that were used to create the mutants. The molecular weight marker lane is omitted. Culture represent by lane 4 was selected.

Table 3.1: Summary of band sizes expected for Southern hybridization results.

Mutant	Enzyme	Wild-type fragment size	Plus pFUS2 (7198 bp)
nadA	PstI	2137	9335
nadB	XhoI	4147	11345
nadC	XhoI	4130	11328
nadA CMD	PstI	2159	9357
nadQ CMD	PstI	1923	9121

IDMs for *nadA*, *nadB* and *nadC* were successfully constructed; however, the *nadA* strain that was used turned out to have a doublet insertion of the vector. While this still rendered the gene non-functional and thus did not invalidate assessment of its SIM function, it made it inappropriate for use in gene expression studies and thus was omitted from those experiments. Likewise, while the *nadQ* IDM was initially thought to be successful, Southern hybridization showed it had not inserted correctly (results not shown) and so the putative mutant was omitted from all results.

In addition to the to the Southern hybridisations, the three mutants expected to be nicotinic acid (NA) auxotrophs (inserts in *nadA*, *B* and *C*) were unable to grow on GRDM that did not contain nicotinic acid, confirming their auxotrophy.

3.5.2 Effects of nicotinic acid on *nad* mutants

As mutants in the *nad* locus were now available, a plating experiment similar to the one described in section 3.4 was undertaken. In this case the *nadA*::pFUS2 mutant was used in the main plating experiment, with R7ANS and R7AΔ*dct* used as reference/control strains. As the previous experiment showed that there was very little difference between the two high and two low concentrations of nicotinic acid used, only concentrations of 1 and 50 µg/ml of nicotinic acid were used. As *nadABC* are expressed as a single transcript, the pFUS2 mutants of both *nadB* and *nadC* were streaked on plates of both nicotinic acid concentrations to see if they showed a similar phenotype to that seen in the *nadA* mutant in the plating experiment.

The results (Figure 3.17) showed that addition of surplus exogenous NA had a negative effect on strains carrying a functional *nadABC* locus, in agreement with the work in section 3.4. R7AΔ*dct* showed a ~30% reduction in the total number of Dct^+ colonies. Conversely, the two strains with either absent or disrupted *nad* loci showed an increase in Dct^+ colonies with 50 µg/ml NA with no or few Dct^+ colonies at 1 µg/ml. As in previous experiments, R7ANS showed virtually no Dct^+ colonies at 1 µg/ml, but the mutator phenotype was partially recovered with the addition of excess NA. The R7AΔ*dctnadA*::pFUS2 mutant showed a similar phenotype but with two differences; at 1 µg/ml it showed some Dct^+ colonies, albeit at a low level and it produced more than twice the number of Dct^+ colonies as R7ANS at 50 µg/ml. However, even when grown at 50 µg/ml NA it still lagged ~30% behind R7AΔ*dct*at 1

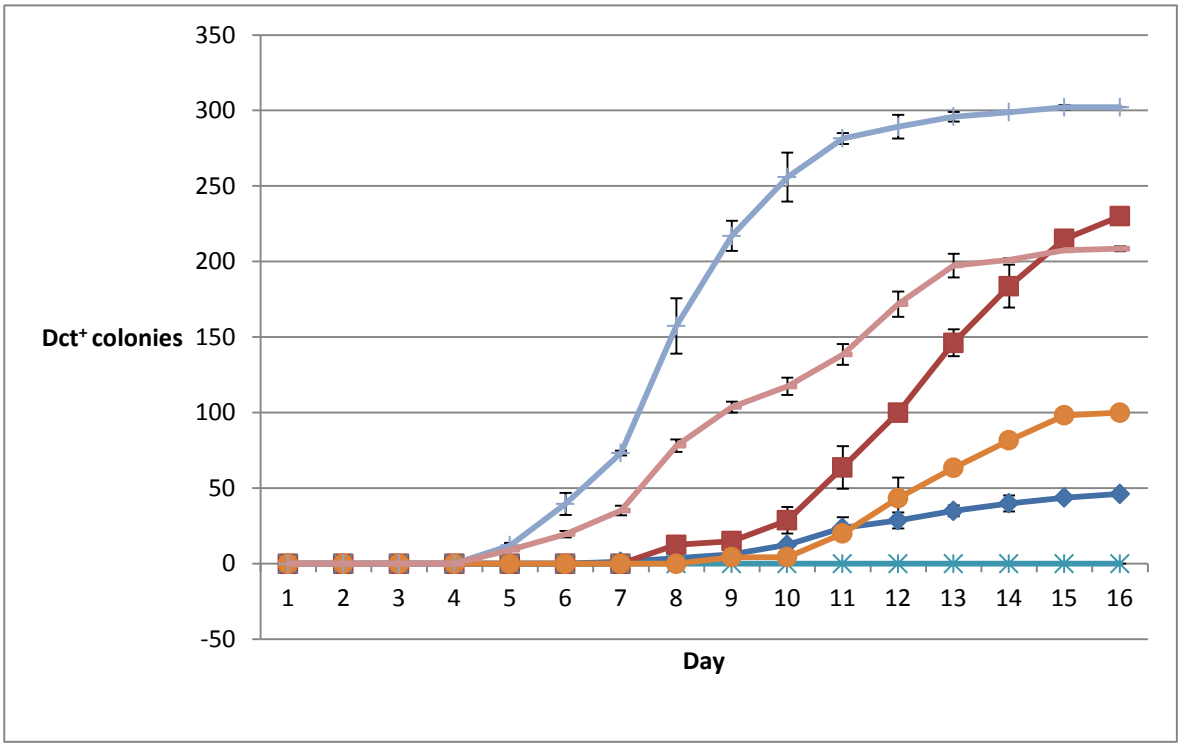


Figure 3.17: Appearence of Dct^+ colonies over time on S/RDM plates containing either 1 or 50 µg/ml of nicotinic acid and inoculated with a 10^{-1} dilution of cell culture. Dark blue diamonds represent R7AΔ*dct nadA*::pFUS2 grown with 1 µg/ml nicotinic acid, maroon squares represent R7AΔ*dct nadA*::pFUS2 grown with 50 µg/ml nicotinic acid, teal asterisks represent R7ANS/pLAFR1 grown with 1 µg/ml nicotinic acid, orange circles represent R7ANS/pLAFR1 grown with 50 µg/ml nicotinic acid, light blue crosses represent R7AΔ*dct* grown with 1 µg/ml nicotinic acid and pink dashes represent R7AΔ*dct* grown with 50 µg/ml nicotinic acid.

µg/ml NA, indicating that high exogenous NA partially but not fully restored the mutator phenotype in the NAD auxotrophic symbiotic strain.

Though not quantified as they were streaked and thus the initial inoculum titre could not be factored in, both the R7AΔ*dct nadB*:: pFUS2 and R7AΔ*dct nadC*:: pFUS2 mutants gave similar results to those observed with R7AΔ*dct nadA*:: pFUS2.

3.5.3 Rate of *nadA::*pFUS2 construct dropout

In the previous experiment there appeared to be some low level mutation occurring in the R7AΔ*dct nadA*::pFUS2 at 1 µg/ml of nicotinic acid, despite the fact that this strain was a confirmed auxotroph and thus should have behaved as R7ANS. As no antibiotics were used in that particular experiment it was postulated that the removal of antibiotic selection may have allowed the pFUS2 insert to 'drop out' restoring the *nadA* gene and therefore restoring autotrophy and by consequence the mutator phenotype. To test this, R7AΔ*dct nadA*::pFUS2 was plated out on two set of plates in tandem, one set containing gentamicin and the other set with no antibiotic. The results showed that when nicotinic acid concentrations were 1 µg/ml, which is too low to induce SIM in NAD auxotrophs, the removal of antibiotics allowed for a small increase in the number of Dct^+ colonies appearing (Figure 3.18a). Though comprising only a few colonies on the high cell density plates, they were significant as when antibiotics were present no Dct^+ colonies appeared. As gentamicin resistance is conferred by the pFUS2 insert, it is likely that this ability to form colonies is due to the loss of the insert and reactivation of the *nad* locus (an event which is lethal in the presence of gentamicin).

At 50 µg/ml NA the difference between the gentamicin and non-gentamicin plates was far less pronounced than that seen at 1 µg/ml and was not consistent across the dilution series, which suggests that though there was loss of the pFUS2 vector when selection was removed, the effect on SIM was negligible.

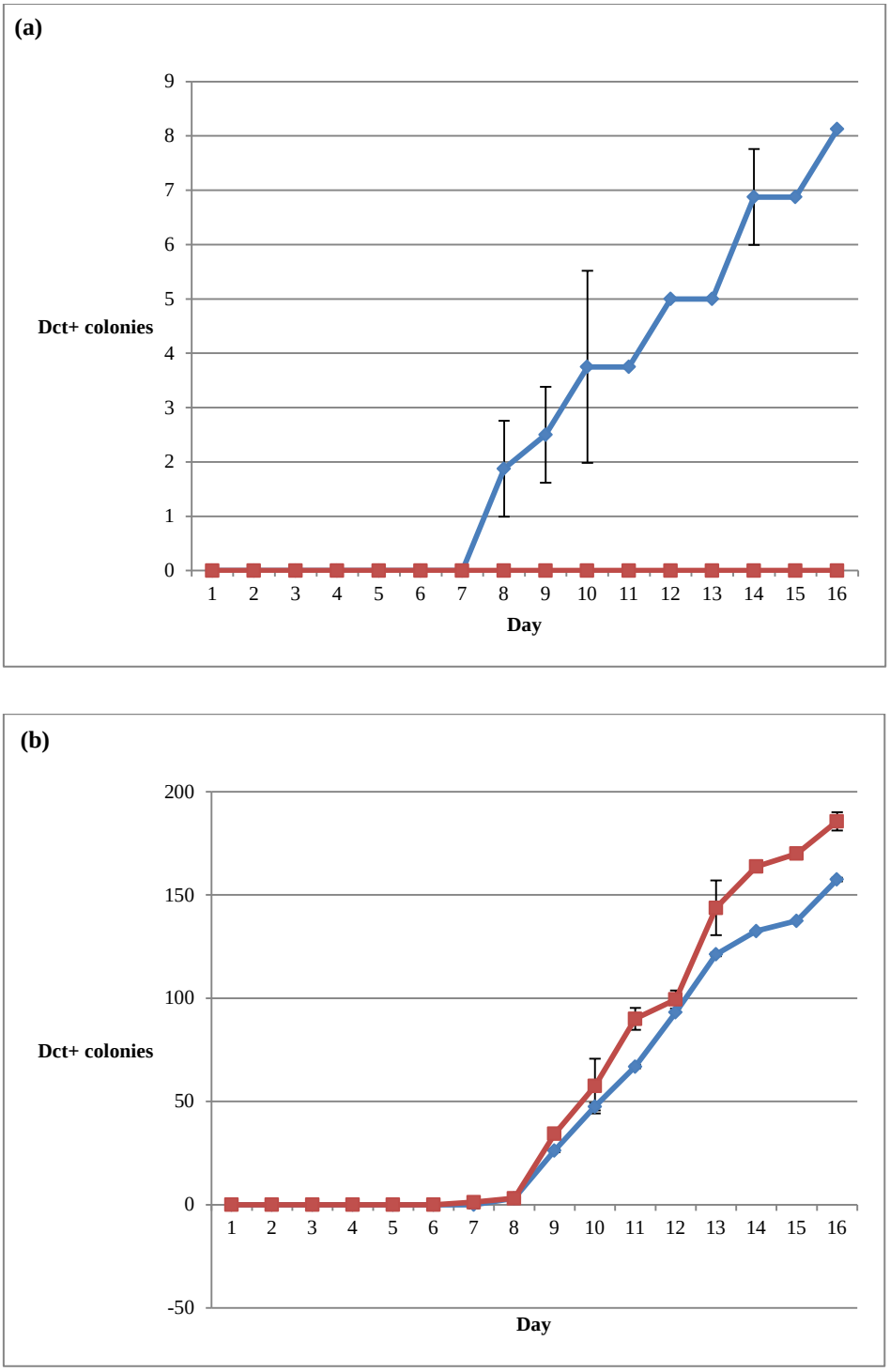


Figure 3.18: (a) Appearance of Dct^{+} colonies on S/RDM with 1 µg/ml nicotinic acid and inoculated with 10^{-1} dilution of R7AΔ*dct nadA*::pFUS2. (b) Appearance of Dct^{+} colonies on S/RDM with a 50 µg/ml concentration nicotinic acid and inoculated with R7AΔ*dct nadA*::pFUS2. Blue diamonds represent plates containing no antibiotics while maroon squares represent those grown on plates containing gentamicin.

3.5.4 Complementation experiment

During the course of several experiments, observations indicated there may be a quantifiable difference in the frequency of mutation to Dct^+ between R7ANS containing the original cosmid p686, R7ANS with the *nadQABC* plasmid p994 plasmid and R7AΔ*dct*. In particular, when p994 was used, the largest increase in the number of Dct^+ colonies was delayed by several days compared to the other strains and far fewer colonies appeared. The differences were quantitated using the standard plating experiment protocol. p994 and p686 were mated into R7ANS and R7A Δ*dct nadA*::pFUS2 backgrounds, with R7AΔ*dct* acting as a positive control. As the two different backgrounds required different antibiotics, plating series were done with and without the required antibiotics to examine whether they introduced any bias. The presence or absence of antibiotics increased and decreased colony numbers depending on the strain being used. This would indicate that these differences were primarily from a selection bias rather than a growth bias; thus media using full antibiotic selection would produce the most accurate results.

Nevertheless, the results of this experiment showed that when p686 was introduced to either background, it gave a dramatic increase in the number of Dct^+ colonies produced compared to the same strain containing p994 and also the R7AΔ*dct* control strain. The plating efficiency of all strains was similar as assessed by the total number of colonies formed. In both strain backgrounds, p686 gave an ~3-fold increase in total number of Dct^+ colonies by the end of the experiment over strains containing p994 and an ~50% increase over the R7AΔ*dct* control (which in turn was 2 fold higher than either p994 strain). Furthermore, p994 strains showed a lag of 1-2 days in initial colony appearance when compared to the p686-containing strains and R7AΔ*dct* (Figures 3.19, 3.20 and 3.21).

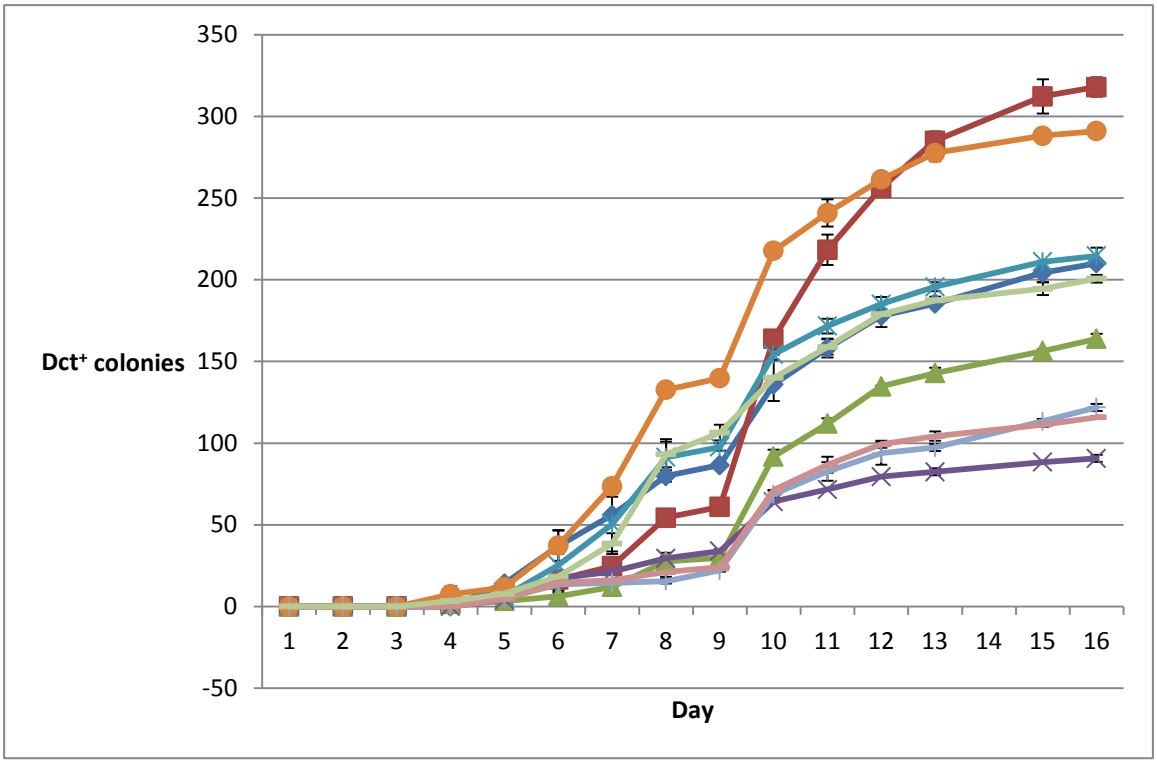


Figure 3.19: Appearance of Dct^{+} colonies after several days on plates inoculated with 10^{-1} dilutions of TY cultures of several strains. Dark blue diamonds represent R7AΔ*dct nadA*::pFUS2 with p686, maroon squares represent R7AΔ*dct nadA*::pFUS2 with p686 grown on tetracycline and gentamicin, dark green triangles represent R7AΔ*dct nadA*::pFUS2 with p994, purple tick and crosses represent R7AΔ*dct nadA*::pFUS2 with p994 grown on tetracyline and gentamicin, blue asterisks represent R7ANS/p686, orange circles represent R7ANS/p686 grown on tetracycline, light blue crosses represent R7ANS/p994, pink dashes represent R7ANS 994 grown on tetracycline and light green represents R7AΔ*dct*.

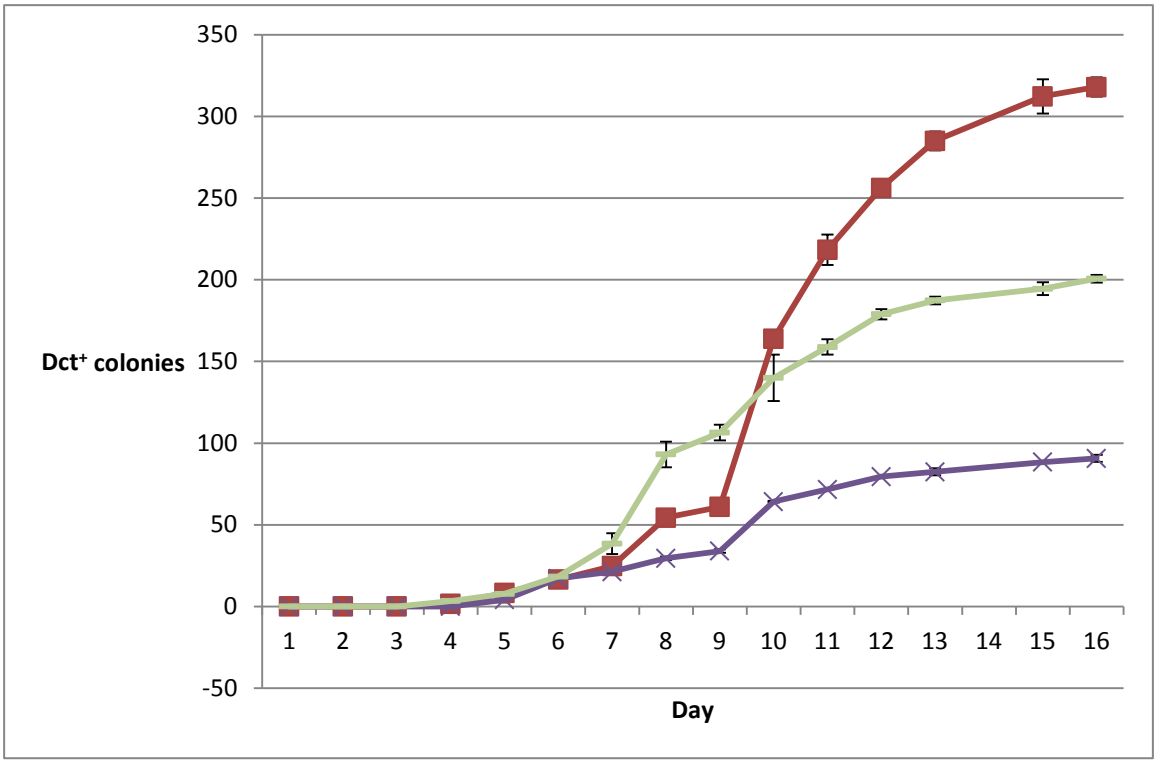


Figure 3.20: Breakdown showing the appearance of Dct^{+} colonies in the *nadA::*pFUS2 strains compared to R7AΔ*dct*. Maroon squares represent R7AΔ*dctnadA::*pFUS2/p686 grown on tetracycline and gentamicin. Purple tick and crosses represent R7AΔ*dct nadA::*pFUS2/p994 grown on tetracycline and gentamicin. Green dashes represent R7AΔ*dct*.

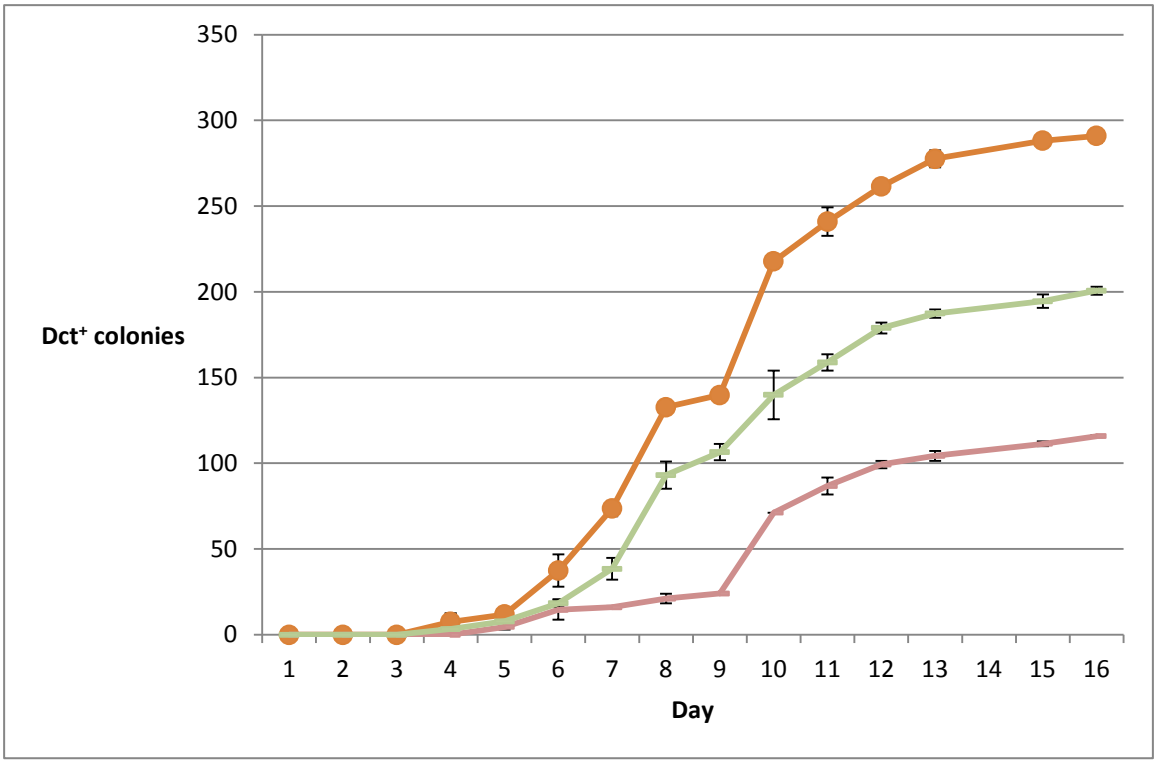


Figure 3.21: Breakdown of the data showing the appearance of Dct^+ colonies in the R7ANS strains compared to R7AΔ*dct*. Orange circles represent R7ANS/p686 grown on tetracyline. Pink dashes represent R7ANS/p994 grown on tetracyline and green dashes represent R7AΔ*dct*.

3.6 Catalase experiment

One particular model being proposed was that the increase in nicotinic acid production leads to a change in the equilibrium between NAD/NADH and NADP/NADPH, causing an upsurge in the relative amount of NADH and promoting the generation of ROS, as will be elaborated further later. This was further highlighted by the phenotype observed in theR7ANS/p994 strain in which at dilutions of high cell density what appeared to be zones of inhibition initially formed around what seemed to be 'pre-Dct^+ colonies' i.e. small colonies that arose from the background lawn after 5 days incubation but that, unlike true Dct^+ colonies, did not continue to expand in size. This phenotype was only observed in the first few days of colony appearance after which many of these colonies converted to a normal Dct^+ type colony. As this form of the Dct^+ phenotype only appeared on the high cell-density plates, it was postulated that a build up of ROS, specifically hydrogen peroxide, could be a primary cause of the apparent inhibition. Furthermore, it was hypothesised that the reason this phenotype was not also observed in the R7ANS/p686 strain was that it may possibly contain a functioning catalase from the symbiosis island that helped alleviate the toxic effects produced by ROS.

To test this hypothesis, the effect of adding an exogenous source of catalase to the media (see Materials and Methods) was tested against a control set of plates in which there was no catalase added (Figure 3.22).The results showed that the addition of catalase caused no observable difference between strains R7ANS/p994 and R7ANS/p686, with the number and onset of Dct^+ colonies in the strain with p994 still remaining significantly behind its p686 counterpart, in agreement with previous experiments. However, the addition of catalase did cause a difference in the number of colonies that appeared (Figure 3.22). The addition of catalase reduced the overall number of colonies produced by both the p994 and p686 strains. This difference though slight was consistent, appearing across all four dilutions in both strains. This ranged from a 2% to 19% reduction from the control plates, with an (Table 3.2) average reduction of 11% and 13% for R7ANS/p686 and R7ANS/p994 respectively. As both catalase and non-catalase plates were inoculated at the same time with the same inocula, it is highly unlikely that these differences occurred via chance.

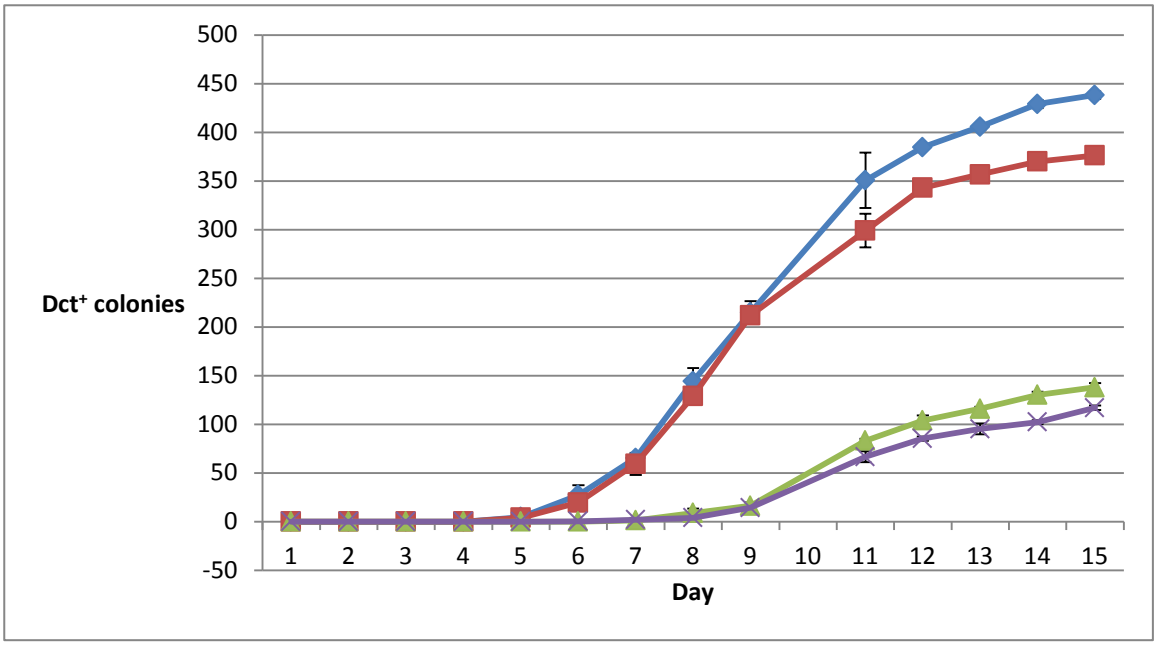


Figure 3.22: Effect of addition of exogenous catalase to the appearance of Dct^+ colonies. Blue diamonds represent R7ANS/p686 while red squares are the same strain grown with catalase. Green triangles represent R7ANS/p994 while purple ticks and crosses are the same strain grown with catalase. Graph shows results from plating a 10^{-1} dilution of both strains.

Table 3.2: Percentage of total Dct^+ colonies that appeared on catalase-treated plates compared to those grown without catalase at the four dilutions of original culture

	10^0	10^{-1}	10^{-2}	10^{-3}	mean
R7ANS/p686	89.2	85.9	88.8	92.7	89.1
R7ANS/p994	85.4	84.8	80.9	97.6	87.2

3.7 *nad* locus regulation

3.7.1 Regulation over time

The pFUS2 vector used to create the IDM and CMD constructs in the *nadQABC* locus creates a transcriptional fusion to the *lacZ* gene. Hence these mutants can be used to assay the level of activity of the gene in which the vector has been inserted using ß-galactosidase assays. Three time points were chosen to examine the expression of each gene, 24, 72 and 144 hours after inoculation. These time points were chosen in an attempt to capture levels of expression at different time points relevant to the progression of the mutator phenotype. The 24 hour time point is when the cells are still undergoing exponential growth and are thus not under limiting conditions. At 72 hours the cells in suspension would have entered stationary phase while at 144 hours the cells have been in a prolonged stationary phase. While cells grown in broths will likely not behave exactly as those grown on solid media, for the purposes of this study it was viewed as a close approximation. Two concentrations of nicotinic acid, 1 and 50 µg/ml, were also used with each strain.

Six strains were used in this experiment. These included R7AΔ*dct nadA*CMD and R7AΔ*dct nadQ*CMD, which contain an in-frame pFUS2 insertion in their respective upstream promoter regions and 5-prime region of the gene (thus still maintaining gene function), and R7AΔ*dctnadB*::pFUS2 and R7AΔ*dct nadC*::pFUS2 which are auxotrophic IDM mutants. The use of R7AΔ*dct nadA*::pFUS2 in this experiment was decided against as Southern hybridization revealed that it contained a doublet insert. However, as *nadABC* are produced as a single transcript the two other IDM strains should provide an accurate approximation of *nadA* activity. The final two strains used were a *nadB* IDM mutant in an R7AΔ*dct*Δ*nadQ*background (provided by John Sullivan) and the control strain R7AΔ*dct*.

The results (Figures 3.23 to 3.25) showed that for the *nadA*-CMD, *nadB*-IDM and *nadC*-IDM strains, that expression at 24 hours was roughly 50-100 Miller units when grown in 1 µg/ml nicotinic acid. At 72 hours expression rose to 200-500 Miller units and at 144 hours it was reduced to 200-300 Miller units. When grown with 50 µg/ml NA, the expression at 24 hours remained similar at ~50 Miller units but as time progressed it dropped steadily such that by 144 hours expression was negligible at <20 Miller units. All three strains showed a high degree of variability as can be seen in the sometimes large standard deviation, but taken together they showed a consistent expression pattern.

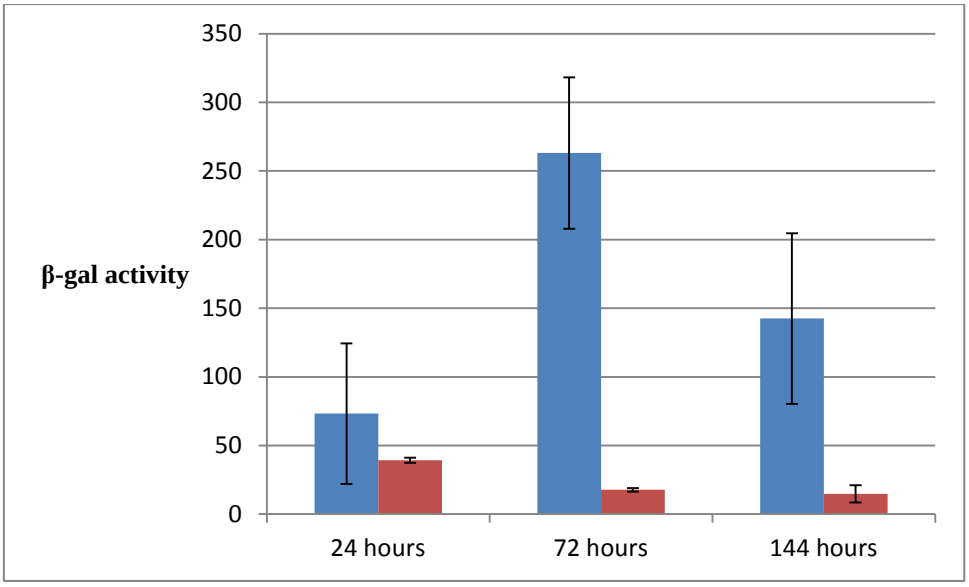


Figure 3.23: actosidase expression of the *nadA* cis-mero diploid construct over time when grown in G/RDM broth containing different concentrations of nicotinic acid. Blue represents 1 µg/ml while red represents 50 µg/ml of nicotinic acid.

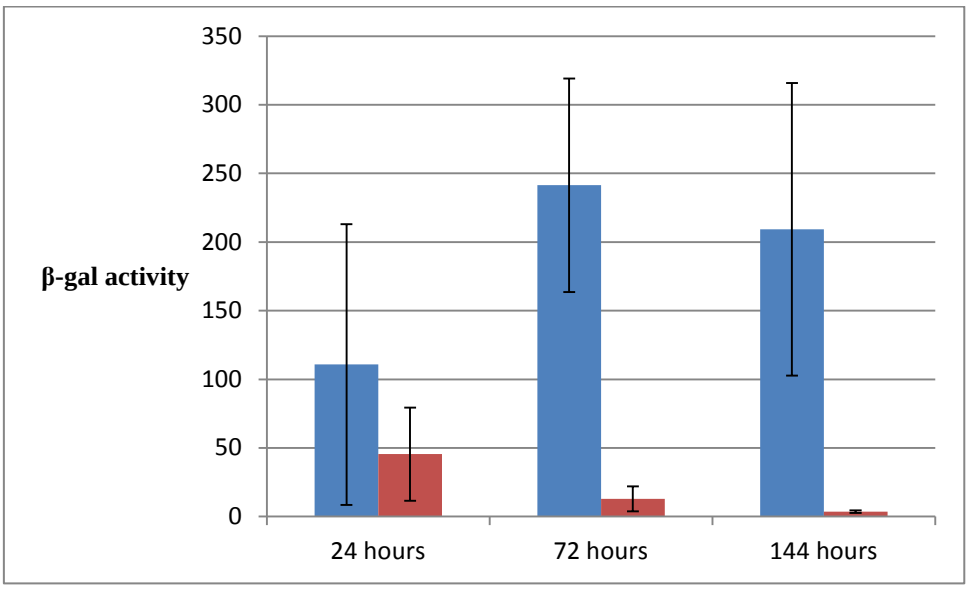


Figure 3.24: β-galactosidase expression of the *nadB*::pFUS2 construct over time when grown in G/RDM broth containing different concentrations of nicotinic acid. Blue represents 1 µg/ml while red represents 50 µg/ml of nicotinic acid.

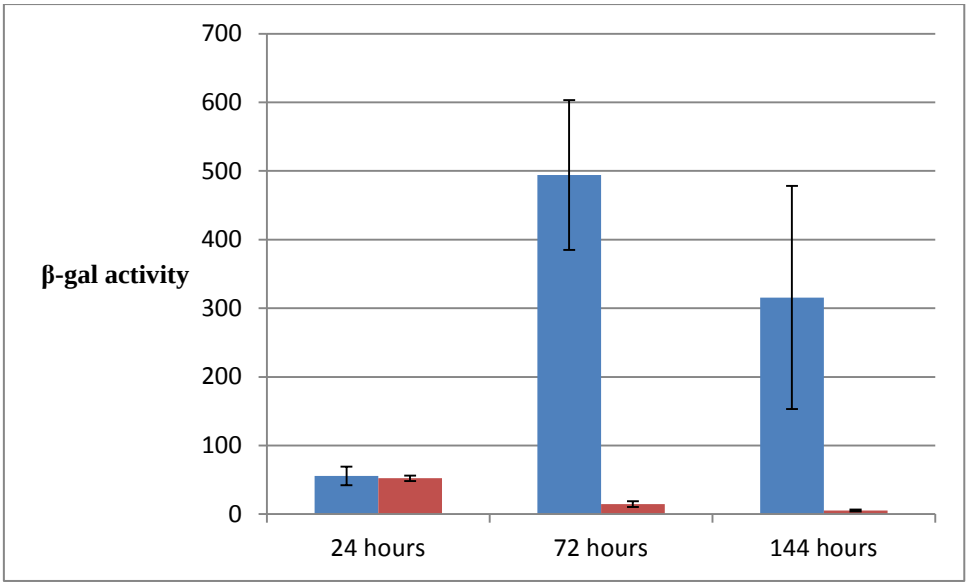


Figure 3.25: β-galactosidase expression of the *nadC*::pFUS2 construct over time when grown in G/RDM broth containing different concentrations of nicotinic acid. Blue represents 1 µg/ml while red represents 50 µg/ml of nicotinic acid.

The LacZ expression of the *nadQ*CMD construct after 24 hours was ~40 Miller units when grown at 1 µg/ml nicotinic acid. Expression remained largely the same at 72 hours, with a slight drop at 144 hours which may reflect natural variation as the means of both the 24 and 144 hour samples fell well within each other's standard deviation. When grown in 50 µg/ml of NA, the pattern of expression was similar to that with 1 µg/ml (Figure.3.26).

The *nadB*::pFUS2 construct, when placed in a Δ*dct*Δ*nadQ* background, showed an increase in expression compared to that observed in the same mutant in the Δ*dct* background. Under all conditions expression exceeded 150 Miller units, with 200-300 Miller units generally seen (Figure 3.28).

3.7.2 Regulation at differing levels of nicotinic acid

To further examine the regulation of the components of the *nad* locus, understanding at what level exogenous NA began to assert repression was deemed necessary. In this set of experiments strains were grown in broth for 72 hours under varying levels of nicotinic acid, starting at 0 µg/ml and increasing in increments of 5 µg/ml to 50 µg/ml. The 72-hour time point was chosen as it was when the cells initially enter stationary phase which may be of relevance to the mutator phenotype. The NA increments were chosen as they provided a reasonably wide range and as seen in previous work there did not appear to be a great effect on the mutator phenotype when NA concentrations were increased above 50 µg/ml.

Three strains were chosen, R7AΔ*dct nadA*-CMD, R7AΔ*dct nadQ*-CMD and R7A. Only R7AΔ*dct nadA*-CMD was selected because, as expected, previous experiments indicated that its expression pattern was consistent with the other two *nadABC* mutants. R7A was used as a control as it contains no *lacZ* gene and would indicate what background β-galactosidase activity R7A produced. Expression of the *nadA*-CMD construct was greatly influenced by the presence of NA (Figure 3.29). When no NA was present an average of 405 Miller units was produced, somewhat higher than that seen in the previous set of experiments where with 1 µg/ml at 72 hours, 263 Miller units were obtained for the *nadA*-CMD construct. When the NA concentration was increased to 5 µg/ml, a sharp decrease in expression to 200 Miller units was observed. After this first increment, further increments show a more linear decline in expression, dropping an average of ~20 Miller units per increment showing about 78% of

the previous increment's expression on average until reaching an average expression of 18 Miller units by the time the concentration of 50 µg/ml was reached (a value consistent with the

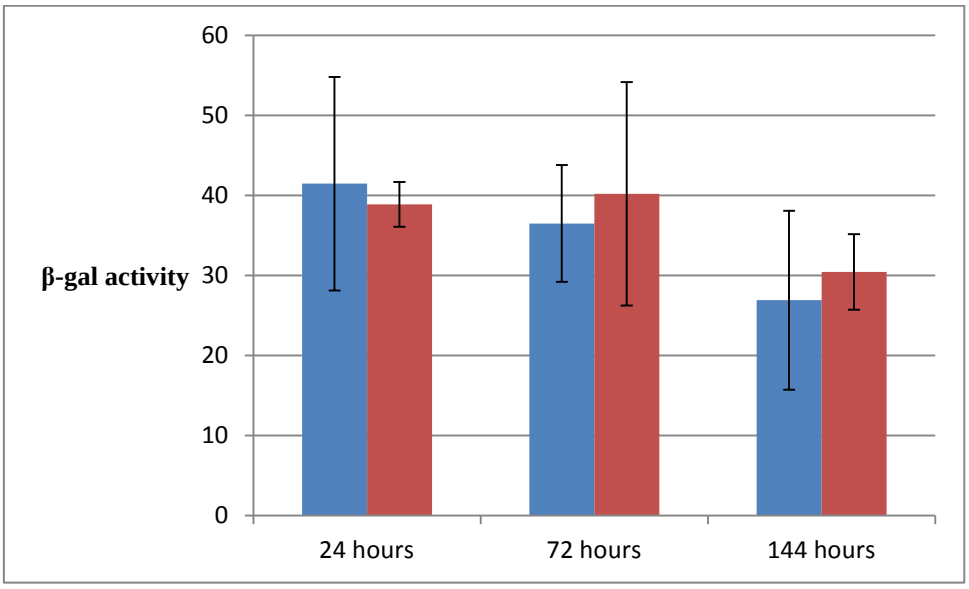


Figure 3.26: β-galactosidase expression of the *nadQ* cis-mero diploid construct over time when grown in G/RDM broth containing different concentrations of nicotinic acid. Blue represents 1 µg/ml while red represents 50 µg/ml of nicotinic acid.

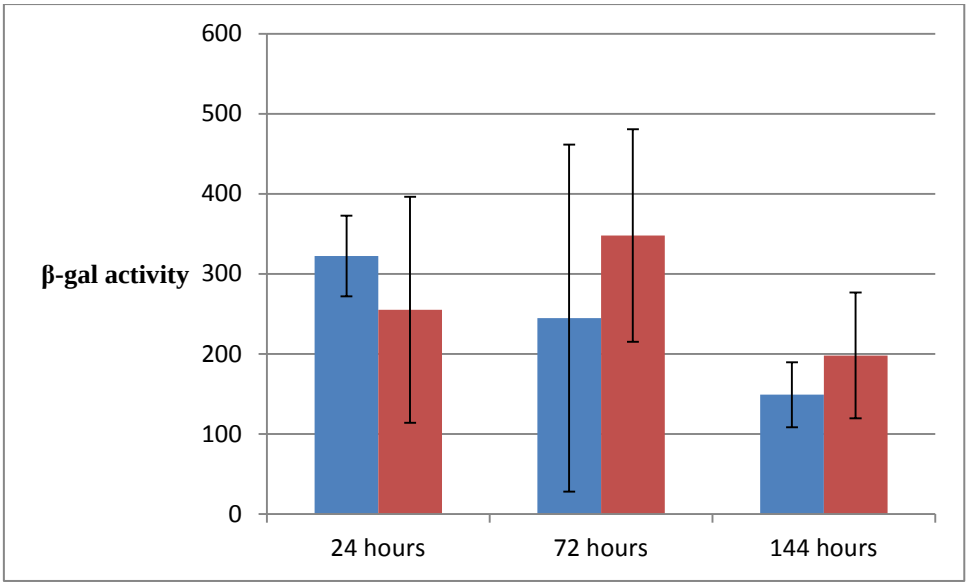


Figure 3.27: β-galactosidase expression of the R7AΔ*dct*Δ*nadQ nadB*::pFUS2 over time when grown in G/RDM broth containing different concentrations of nicotinic acid. Blue represents 1 µg/ml while red represents 50 µg/ml of nicotinic acid.

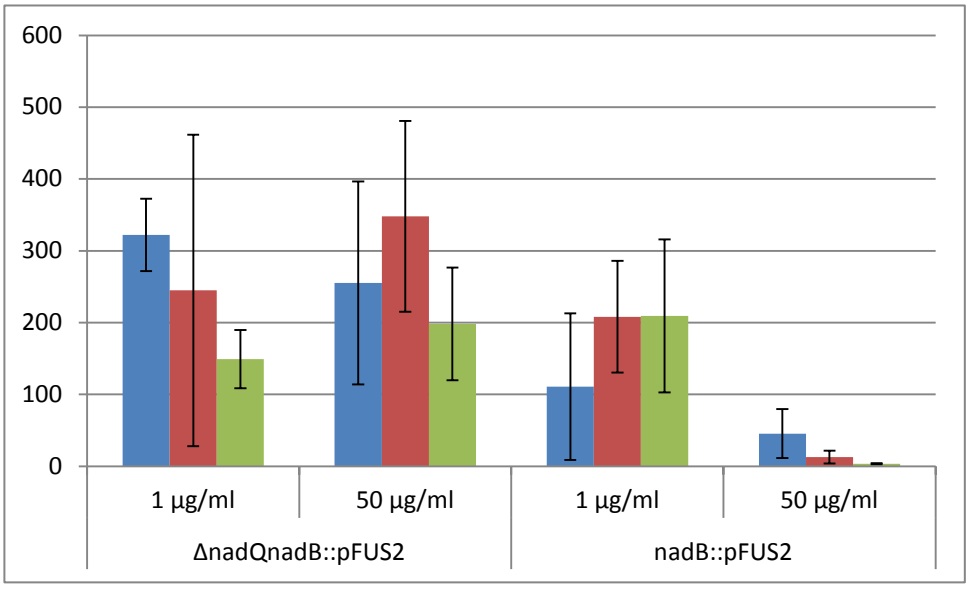


Figure 3.28: Comparison between β-galactosidase expression profiles of *nadB*::pFUS2 in either R7AΔ*dct* or R7AΔ*dct* Δ*nadQ* backgrounds. Blue represents 24 hours, red represents 72 hours and green represents 144 hours.

previous experiment). Though the overall trend showed a decrease with each increment, there was an uncharacteristic rise between 25 and 30 µg/ml. As the standard deviations for these two data points were quite large, it is likely that this anomaly arose from chance biological variation in expression and sensitivity of the assay and would likely disappear if further replicates were carried out.

The expression pattern for the *nadQ*CMD fusion was unaffected by the concentration of NA in the media, as seen in previous experiments. All values fell between 44 and 56 Miller units, with an average of 49.5 across all concentrations with a standard deviation of 3.4 (Figure 3.30). Lastly, the expression of the R7A control showed very little activity at any concentration of nicotinic acid. At no point was expression equal to or greater than one Miller unit, with many data points giving no reading (data not shown).

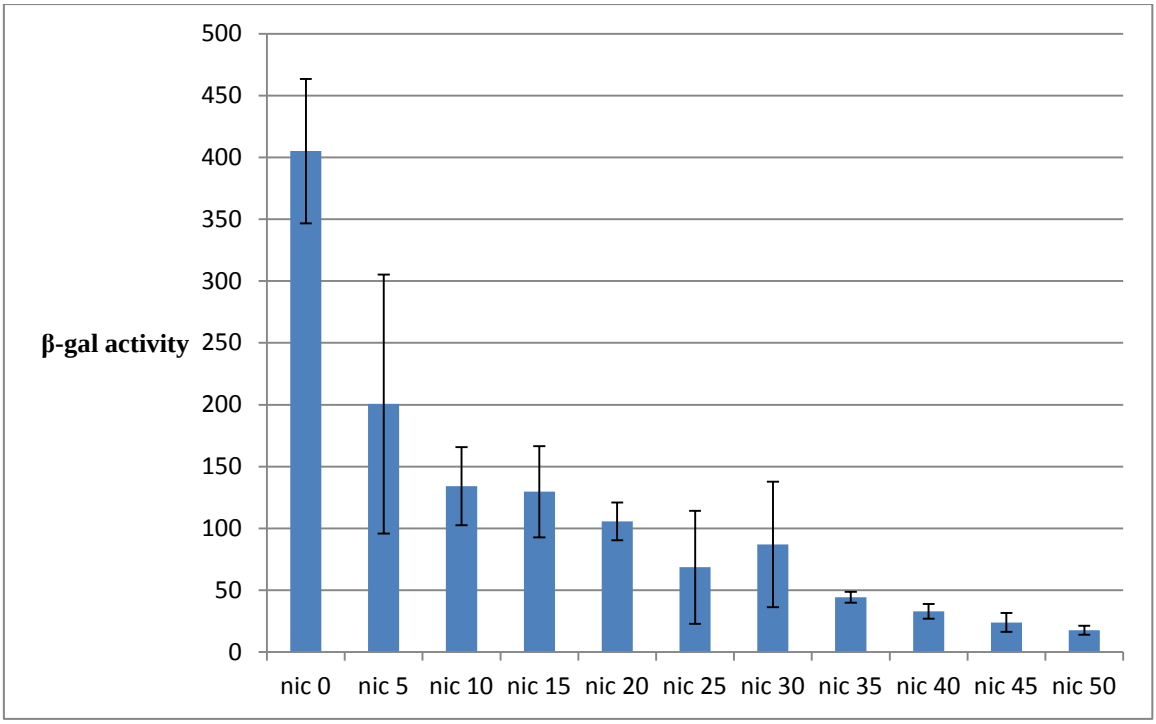


Figure 3.29: β-galactosidase activity of *nadA*CMD construct at 72 hours at differentconcentrations of nicotinic acid (µg/ml).

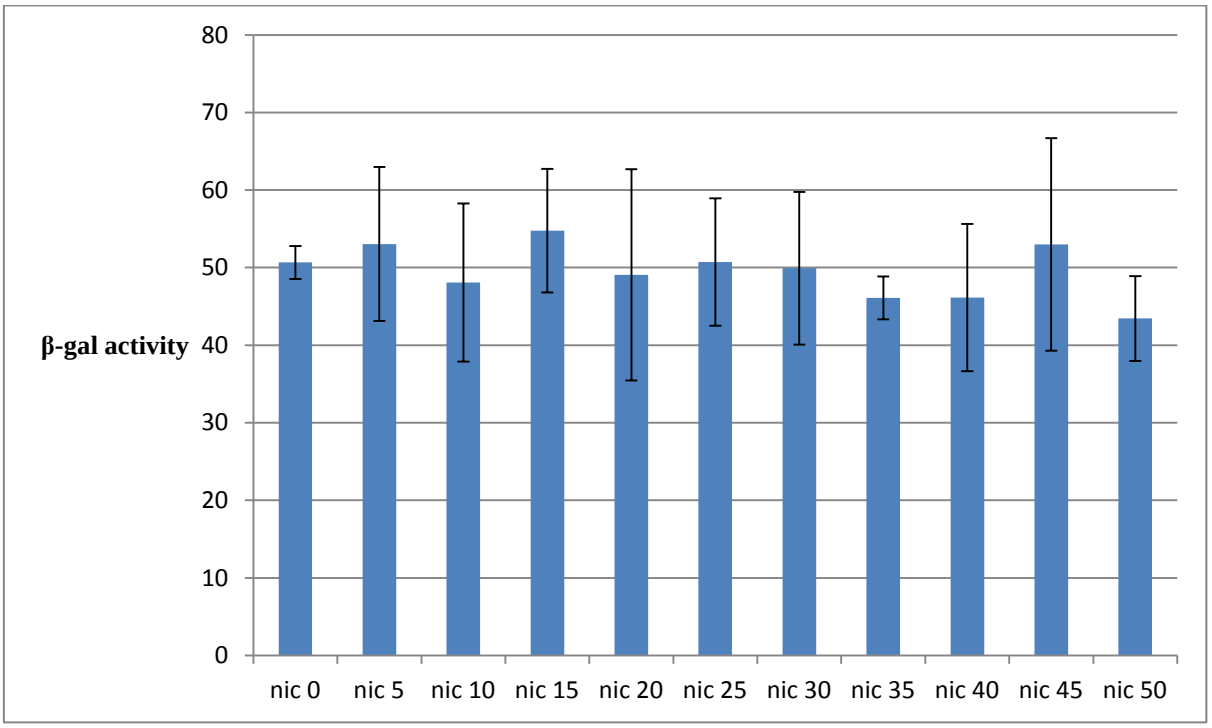


Figure 3.30: β-galactosidase activity of *nadQ*CMD construct at 72 hours at different concentrations of nicotinic acid (µg/ml).

3.8 Mechanisms

Previous work had indicated that Mfd, a transcription-coupled repair protein, had a limited effect on the mutator phenotype in strains containing pJW5 but this had not been fully quantified. Previous work had also indicated that *recA*, an important gene associated with double strand break repair, appeared to have no effect on the mutator phenotype, but again this was not quantified. Thus, determination of whether mutations in either of these genes had a quantifiable effect on the frequency of Dct^+ colonies should provide useful information on the repair mechanisms involved with the mutator phenotype and thereby provide insight into the mechanism behind the phenotype as a whole. Mutants in both genes were already available in an R7ANS background and were used for the experiments.

The first experiment was a plating experiment similar to the ones described above, in which strains R7ANSΔ*recA* and R7ANSΔ*mfd* were grown on succinate media containing either 1 or 50 µg/ml of nicotinic acid, with R7ANS/pLAFR1 and R7AΔ*dct* being used as negative and positive controls respectively. The results showed that both the *mfd* and *recA* mutants behaved in a manner similar to R7ANS/pLAFR1, both in terms of growth curve and the overall number of colonies produced. At 1 µg/ml of NA, virtually no Dct^+ colonies appeared in any of the nonsymbiotic strains and R7AΔ*dct* behaved as expected. At 50 µg/ml, all three nonsymbiotic strains showed growth of Dct^+ colonies consistent with previous results. R7ANS/pLAFR1 showed slightly higher overall number of colonies than either the Mfd or RecA-deficient strains, but the difference was small and well within the standard deviation of the strains (Figure 3.31).

A second experiment was done in a similar way to the complementation experiment undertaken in section 3.5.4. In this case p994 and p686 were each mated into R7ANSΔ*mfd* and R7ANSΔ*recA*.The results for this experiment showed a wide deviation from both the immediately previous experiment, but also other experiments carried out with some of these strains. In particular the R7ANSΔ*recA*/p686 strain showed a significant increase in Dct^+ colonies, which was unexpected. Also of note was that the p994 strains showed figures close to or exceeding those seen in R7AΔ*dct*, including the R7ANS/p994 control, with only the R7ANSΔ*mfd*/p994 strain showing a figure similar to the usually relatively depressed number of Dct^+ colonies seen in R7ANS/p994. These two results point to some form of systematic error in the experiment, most likely a simple error in the dilution series. For this reason, the experiment must be repeated. Unfortunately, this was not possible within the time frame of

this study. However, the experiment is included here, as it clearly supports one of the findings from the previous experiment i.e. that Dct^+ colonies formed in strains deficient in either Mfd or RecA when the *nad* genes are present.

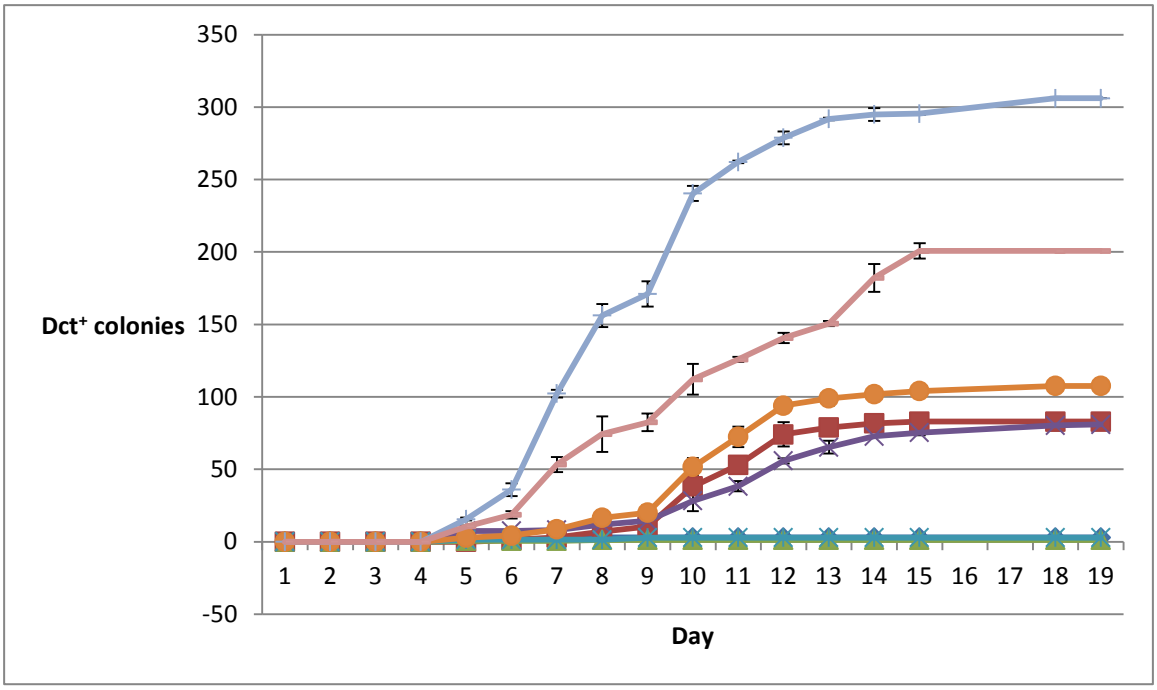


Figure 3.31: Appearance of Dct$^+$ colonies over time in Mfd and RecA-deficient R7ANS strains, when grown on media containing either 1 or 50 µg/ml nicotinic acid. Blue diamonds represent R7ANSΔ*mfd* grown at 1 µg/ml, maroon squares represent R7ANSΔ*mfd* grown at 50 µg/ml, green triangles represent R7ANSΔ*recA* grown at 1 µg/ml, purple cross and ticks represent R7ANSΔ*recA* grown at 50 µg/ml, teal asterisks represent R7ANS/pLAFR1 grown at 1 µg/ml, orange circles represent R7ANS/pLAFR1 grown at 50 µg/ml, light blue crosses represent R7AΔ*dct* grown at 1 µg/ml and pink dashes represent R7AΔ*dct* grown at 50 µg/ml.

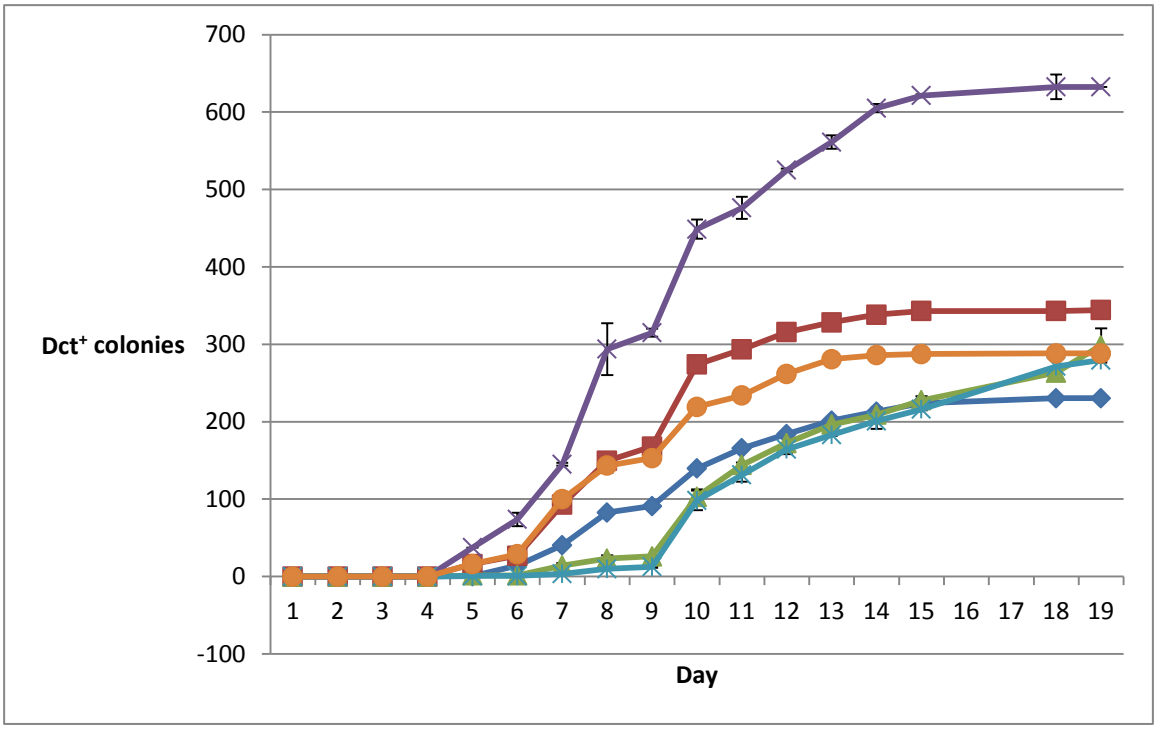


Figure 3.32: Appearance of Dct$^+$ colonies over time in Mfd and RecA deficient strains, in an R7ANS background, when complemented with either of the *nad* locus plasmids, p686 or p994. Blue diamonds represent R7ANSΔ*mfd*/p994, maroon squares represent R7ANS/p686, green triangles represent R7ANS/p994, purple cross and ticks represent R7ANSΔ*recA*/p686, teal asterisks represent R7ANSΔ*recA*/p994 and orange circles represent R7AΔ*dct*.

4. Discussion

4.1 R7A succinate reversion

The mutator phenotype in *M. loti* as displayed by the activation of the cryptic chromosomal *dct* genes presents several unique and contrasting features that yield insight into the nature of the phenotype. Firstly the non-linear appearance of Dct^+ colonies over a range of dilutions comprises a key informative feature. The explanations as to why a 10-fold decrease in the number of cells plated does not in turn lead to a 10-fold decrease in the number of Dct^+ colonies are several and not necessarily mutually exclusive. The first of these could simply be a case of exhaustion of the medium. Particularly at the very early dilutions such as 10^0 and 10^{-1}, the number of Dct^+ colonies is so great that they cover an extensive area of the plates and it is likely that these dilutions reach an upper limit of Dct^+ colonies that the nutrients in the agar can support. Secondly, the number of Dct^+ colonies is such that physical space on the plates becomes limiting and so early-arising colonies may mask the potential of late-arising colonies to appear. In addition, the total cell population on the plates that subsequent revertants can mutate from does not entirely follow the linear dilution series that was originally plated. This is due to the scavenging of impurities in the agar which allows for limited amounts of replication. At the lower dilutions the original cell density is so great that it is unlikely that much replication occurs due to the intense competition for scant resources. However, at higher dilutions, in which significantly less cells are plated (10^{-7} plates typically had a c.f.u of between 10 and 20), discrete micro-colonies are formed over a longer time frame and the cells are quite likely to have undergone multiple rounds of replication. Indeed, such micro-colonies on the 10^{-7} plates contain between 10^7 and 10^8 bacteria each (J. Weaver and C. Ronson, pers. comm.). Moreover, over a long enough time-frame, over 90% of these colonies will give rise to Dct^+ revertants. Thus limitations in nutrients, physical constraints and the resulting differences in residual growth all likely contribute to the non-linear trend. All of these are artifacts of the method used and were somewhat corrected for in later experiments by the use of more refined Noble agar, but in hindsight the addition of a scavenger culture that lacks the genetic potential to develop Dct^+ revertants may also have been of advantage to estimate the contribution of residual growth to the production of mutants. Nevertheless, at the higher dilutions, Dct^+ colonies only appeared after the micro-colonies had stopped expanding, indicating that most mutations arose in non-growing cells.

Another feature of note was the lag time between the peaks in Dct^+ colony appearance as the dilution series progressed. One would expect this peak in mutants to occur at same time across all the dilution series, with the difference being in the number of mutants appearing at that peak. This is not the case with general trend of a delay of one day as the dilution series progresses, meaning that the plates with the lowest number of cells plated experience their optimum mutation rate well behind that of the highest number of cells plated. The explanation for this is unlikely to be related to exhaustion of succinate as mutants still appear for several days after this peak in all cases, hence there is still a large enough nutritional base to support colony formation. One likely explanation is that the availability of media impurities to fuel residual growth is relative to the density of cells originally plated and thus as the level of dilution increases so will the time period in which cells are able to grow. As cells replicate slowly for longer, there will be a delay before they reach starvation and hence stress, so the staggered mutational optima could be indicative of a staggered entry into starvation. Hence again the kinetics of mutant appearance is consistent with the mutations arising in non-growing cells.

Alternatively, cell density may have a modulating effect on how quickly cells enter an SIM state through interactions with neighbouring cells. Such a process has been suggested in *B. subtilis* in which quorum signaling could lead to differentiation of a HMS (77). Obviously relative cell densities would have effects on the nature, strength and timing of potential cell-to-cell signaling. In the same vein, build-up of toxic cellular byproducts could also have an effect by elevating stress. In particular, the release and absorption of exogenous ROS, principally in the form of H_2O_2, could be directly mutagenic.

Due to the above-mentioned factors, only very crude estimates of how much the rate of mutation is elevated in the R7AΔ*dct* strain under stress can be made. R7ANS typically displays a very low, almost undetectable level of reversion most likely arising from a preexisting mutation so this can tentatively be taken as the base-line rate. If the generous figure of one Dct^+ colony appearing on 10 undiluted R7ANS plates is taken, and presuming that these mutants arise from a similar sized population of cells in both strains, then the addition of the symbiosis island seems to confer a roughly 4000-fold increase in the frequency of mutation, much higher than that observed in the Cairns system (85).

4.2 Identification of p686 and involvement of the *nad* locus

During the course of this work several screens were conducted with cosmid libraries of R7A genomic DNA partially digested with either Sau3A or EcoRI. These screens were conducted on a scale that after four attempts at screening, should have given several-fold coverage of the genome, so why the p686 cosmid was never encountered in the course of the screen is somewhat puzzling. Given the later results, it is likely that because of the proximity of the island *dct* locus to the *nad* locus, many cosmids containing the *nad* locus would have conferred the ability to grow on succinate through the presence of the full island *dct* locus. Due to the repeated failure of the library screens, several other approaches were put forward such as cosmid walking across the island in hope of chancing upon the locus responsible. Luckily however, before this approach was undertaken, a small directed screen of several overlapping cosmids used in the sequencing of one end of the symbiosis island (102)revealed a cosmid that appeared to confer the mutator phenotype, namely p686.This screen included a cosmid that contained the island *dct* locus in its entirety, providing a positive control for succinate utilization. Cosmid p686 contains a 27-kb insert encompassing 26 full annotated ORFs, some confirmed and others hypothetical (Figure 3.4). One feature of the p686 cosmid was that it did contain a significant fraction of the *dct* locus, namely the *dctA* gene and part of *dctB*, and so whether this was responsible for the p686 phenotype needed to be resolved.

Deletion of the island *dctA* gene caused no reduction in the cells ability to grow on succinate, suggesting that the island DctB and DctD proteins were capable of inducing expression of the chromosomal *dctA* gene. While the deletion of the island *dctD* gene did substantially reduce growth, growth was still significant, suggesting that the presence of the island *dctB* gene alone was enough to allow the utilization of succinate. This was confirmed by the observation that the two strains that had deletions of the island *dctB* genes were unable to grow on succinate. Taken together, the results indicate that the chromosomal DctB gene product is defective. This can be rescued by the addition of the island DctB gene product, albeit at a lower efficiency with chromosomal DctD than with island DctD. In conclusion, the requirement for a fully functioning copy of the island *dctB* gene to be present confirmed that what remained of the island *dct* locus on p686 was insufficient to confer an ability to grow on succinate. However, this does not rule out the possibility that the *dct* sequences present on p686 may have some modulating effect on the mutator phenotype.

Having excluded the island *dct* remnants as the sole cause of the mutator phenotype, the question as to what on p686 was leading to Dct^+ colonies remained. The initial approach was to see if there were any genes in common between p686 and the pJW5 region that had already been isolated from the nonsymbiotic strain CJ1. It was found that two loci were in common, *fixV* which encodes LacI-family regulator and the *nad* locus. As plasmids clones were already available that contained these genes, the causative nature of the *nad* locus was quickly established by introducing the plasmids into R7ANS.

Plasmids p994 and p1018 (which was first used to identify the *nad* locus with the mutator phenotype) but not p993 conferred the mutator phenotype. Compared to the other plasmids, p993 contains only a 3-prime portion of the *nadQ* gene suggesting that this gene was essential for the mutator phenotype. The *nadQ* gene was named by Rodionov *et al*. 2008 (78) on the basis of its association with *nad* genes in several bacteria, and is located upstream of the *nadABC* locus with a ~50 bp gap between the end of *nadQ* and the start of *nadA*.

4.3 The role of the *nad* locus and NAD precursors

The *nad* locus encodes three genes (*nadA*, *nadB* and *nadC*) that are probably transcribed as a single transcript, as the stop codon of *nadA* overlaps with the start codon of *nadB*, and the stop codon of *nadB* overlaps with the start codon of *nadC*. Another ORF initiates 11 bp downstream of the *nadC* but this ORF is not required for NAD synthesis and it has no homologues in the databases, suggesting that it may not be a real gene. The *nadABC* genes encode enzymes that comprise the first three steps for *de novo* synthesis of nicotinamide dinucleotide (NAD), in which L-aspartate is first converted to iminoaspartate by the *nadB* gene product. Iminoaspartate is in turn condensed with dihydroxyacetone phosphate by the *nadA* gene product to form quinolinate which in turn reacts with 5-phosphoribosyl-1-pyrophospate as catalyzed by the *nadC* gene product to produce nicotinic acid mononucleotide (NaMN). NaMN is then further transformed to NAD by the *nadD* and *nadE* gene products which act in both the *de novo* and salvage pathways of NAD synthesis and are located elsewhere on the genome (107).

Nicotinamide adenine dinucleotide (NAD) and its reduced and phosphorylated derivatives (NADH, NADP and NADPH) have a ubiquitous role as hydride acceptors and cofactors

involved in cellular metabolism, DNA ligation and protection against oxidative stress among others (36). Due to its vital role in many cell functions and its conserved use across almost all taxa, NAD has become one of the most accurately mapped biosynthetic pathways (5). In both prokaryotes and eukaryotes, NAD is produced through either a *de novo* pathway or through the salvage of either exogenous precursors or endogenous byproducts of NAD breakdown, such as nicotinic acid, nicotinamide, nicotinamide mononucleotide (NMN) and various other molecules (58, 88).

As the *nadQABC* locus identified on p686 and pJW5 encodes enzymes for the first three steps for the *de novo* biosynthesis of NAD, a logical hypothesis is that the mutator phenotype is related to increased synthesis of NAD as neither decreased expression nor maintenance of *de novo* synthesis would explain the need for this locus (all strains in initial work were supplemented with 1 µg/ml nicotinic acid as part of a typical vitamin mixture as most of the nonsymbiotic *M. loti* strains used are NAD auxotrophs (100). The approach of adding varying concentrations of nicotinic acid provided direct evidence that the increased production of NaMN is responsible for the mutator phenotype. Addition of exogenous nicotinic acid at 50 µg/ml or greater initiated the mutator phenotype in nonsymbiotic and NAD auxotrophic strains. While the mutator phenotype was established in the R7ANS strain at these higher concentrations, the mutator phenotype never reached levels achieved when an intact *nadABC* locus was present, indicating that either transport of nicotinic acid into the cell or the conversion of nicotinic acid into the relevant NAD precursor may serve as a limiting factor. This wais further shown by the partial repression of the mutator phenotype in NAD-prototrophic strains by the addition of 50 µg/ml nicotinic acid. It would appear that there is a discrepancy between the amount of nicotinic acid that can be transported into or utilized by the cell, compared to the amount of NaMN or NAD that can be synthesized *de novo*, and that when suppression of the *de novo* process occurs total intracellular NAD concentration is less despite high exogenous concentrations of salvageable precursors.

These experiments clearly demonstrated that high concentrations of nicotinic acid can at least partially replicate the mutator phenotype and, as nicotinic acid is also salvaged to produce NaMN, they provide independent support for the conclusion that the *nad* locus is causing the mutator phenotype. This also eliminates the possibility of one of the intermediates in the *de novo* pathway, such as quinolinate being responsible, as the only common feature between the *de novo* pathway and nicotinic acid salvage is the formation of NaMN. This then raises

the question as to how the production of NaMN leads to the mutator phenotype. As the main biological role of NaMN is to act as a precursor to the synthesis of NAD, this would seem the most likely role.

The experiment examining the level of loss of the pFUS2 insert in the *nadA::*pFUS2 mutant showed that there was indeed loss of the vector when selection was removed and raised questions as to why this occured and at what rate. It would appear to be that when a strong (lethal) positive form of selection in the form of gentamicin was removed, a weaker negative selection back towards nicotinic acid prototrophy came into force. This selection toward nicotinic acid prototrophy would seem unnecessary because the plates contained nicotinic acid (1 µg/ml is a more than adequate amount to satisfy the auxotrophy). However, it would appear to support the body of findings that stationary cells require or attempt to acquire an increased level of nicotinic acid, explaining why positive selection is induced and why this phenomenon is not observed when *nadA*::pFUS2 mutants are grown at nicotinic acid concentrations of 50 µg/ml. The rate at which pFUS2 loss occurred is difficult to ascertain and remains uncertain. It must be kept in mind that the Dct^+ colonies underwent two mutational events, the first the loss of the pFUS2 insert and then the activation of the chromosomal *dct* genes. Thus the number of Dct^+ colonies only represents a detectable subpopulation of the total number of cells that lost their pFUS2 insert, which could potentially be far larger. However, as the original cells plated underwent several rounds of replication during micro-colony formation, attempting to determine the rate of pFUS2 drop-out through inferences based on the number of Dct^+ colonies is an imprecise proposition at best. Also, the Southern hybridization data suggested that the *nadA*::pFUS2 mutant had a double insertion of pFUS2 and therefore this may have contributed to the instability of the mutant.

Nevertheless, two important conclusions can be drawn. The first is that while pFUS2 loss does occur, it was negated by the maintenance of antibiotics in the media. Secondly, the presence of even a small positive selection back to nicotinic acid prototrophy during stationary phase, even when ample nicotinic acid is available in the medium for normal growth, is a supporting piece of evidence for the increased need for nicotinic acid in stationary phase and its facilitation of the mutator phenotype.

A general observation was that p994, when added to a nonsymbiotic strain led to the appearance of substantially fewer Dct^+ colonies compared to both the original R7AΔ*dct*

mutant and non-symbiotic strains containing p686. The most obvious reason would be that, as the only difference between p994 and p686 is that p686 contains additional genes, some of the additional genes may have a pleiotropic effect, in that they encourage the formation of Dct^+ colonies through improved growth or survivability. The region of the symbiosis island that is encompassed by p686 contains many hypothetical open reading frames with no assigned function. Genes that increase the growth rate of cells, such as those that may allow more effective scavenging of media impurities for example, would lead both to a faster build-up of cells and exhaustion of nutrients (thus earlier entrance into stationary phase) and also possibly a larger population of cells for Dct^+ mutants to arise from. This could both explain the delay seen in p994 and the lower number of Dct^+ colonies. Also, genes that promote survivability could be involved as they would increase the population of cells able to persist on the media for longer, thus increasing the population of potential mutants at any particular time point. These survivability effects may also be related to the possible negative effects associated directly with the increased mutagenic state. The buildup of ROS during stationary phase is well documented (50, 56), and while being mutagenic it also has major negative effects on general cell health. In particular, the possible involvement of catalase/peroxidase in ameliorating the effects of ROS both as a general mechanism and in relation to both p994 and p686 will be discussed in more depth later on.

One observation argued against the involvement of unknown 'secondary' genes on p686. This was that the addition of p686 to either R7ANS or R7AΔ*dctnadA*::pFUS2 led to an increase in Dct^+ colonies, not just in relation to p994 but also in relation to the original R7AΔ*dct*. R7AΔ*dct* contains all these genes as it carries the complete symbiosis island and so while it does have greater mutability than R7ANS/p994, it should not lag behind R7ANS/p686. It is conceivable that as this region is now cosmid-borne in R7ANS/p686, it exists at a higher copy number per cell whereas in R7AΔ*dct* it exists as a single effectively chromosomal copy on the integrated symbiosis island. Additionally, the genes on p686 could be expressed at a low level by read-through from a vector promoter. If the increased number of copies or expression of this hypothetical gene(s) lead to an increase in the original advantage it prescribed, this would therefore also lead to an increase in the number of mutants.

A second mechanism that may be responsible is based on the genes being used to observe the mutator phenotype rather than any overall physiological effect. In this case it is possible that

the remaining *dct* locus sequences located on p686 may be responsible. In this case it is proposed that these remaining *dct* genes do not act directly to produce a partial Dct^+ phenotype but rather improve the ability of Dct^+ mutants to form. For example, having extra functional copies of *dctD* and *dctA* may provide a larger target for mutation to occur. Furthermore, the presence of at the very least two *dctA* genes (as it is the transmembrane symporter) may give a further growth advantage when the cells do in fact develop the ability the utilize succinate.

One issue that is still to be resolved is that strains containing p994 lagged behind R7AΔ*dct* even when p994 was added to the R7AΔ*dct nadA*::pFUS2 strain. This should now have the exact same genetic quotient as R7AΔ*dct* except that the *nad* locus is now plasmid borne. A possible explanation is that while the island *nad* locus has now been disabled in R7AΔ*dct nadA*::pFUS2, it still has an intact copy of *nadQ* on the island which has its own independent and apparently constitutive (as will be expanded on later) transcription. It could be that *nadQ* is now produced in excessive amounts due to two copies of the gene leading to increased repression of the *nadABC* locus and thus a lowered mutator phenotype. However, this does not entirely fit as significant repression of the mutator phenotype in NAD prototrophs only seems to occur in conjunction with high concentrations of nicotinic acid (or some other NAD precursor), thus the level of nicotinic acid is usually the deciding factor in suppression, not the levels of NadQ protein. Also if this scenario was correct, one would expect to see similar levels of repression when p686 was added to R7AΔ*dct nadA*::pFUS2, which was not the case as it in fact out-performed its R7ANS counterpart. Furthermore, this was also the case for R7ANS/p994 which showed the lowest number of Dct^+ colonies of the mutator strains examined, despite the fact it would not suffer from this deleterious scenario.

While the quantitative differences between the various mutator strains and cosmids do not affect the overall conclusion that the *nad* locus and its products are the deciding factor in the mutator phenotype, resolution of the reasons behind these apparently contradictory results may help elucidate further the overall model.

One further question addressed in the complementation experiments was the effect of antibiotics in the medium on the mutator phenotype. Depending on the strain, media were prepared with up to two forms of antibiotic. As the resistance to antibiotics often incurs a certain metabolic cost (7) it was surmised that the presence of antibiotics may have had a negative effect on the cells being grown as opposed to strains such as R7AΔ*dct* which had no

such stress. This was largely resolved in that the overall effect of antibiotics appeared to be minimal when comparisons were made. In fact strains requiring the presence of antibiotics generally produced higher numbers of Dct^+ colonies when grown with their full complement of antibiotics. This included the NAD auxotrophic strains, which had shown a selective advantage towards reversion to NaMN prototrophy when the strong selective pressure of antibiotics was removed. This was most likely due to the presence of tetracycline selecting for maintenance of p686, which as it based on pLAFR1 lacks stability genes and thus may suffer significant rates of spontaneous loss (109). Due to this, it can be surmised that the rate of spontaneous loss of p686 is higher than the spontaneous reversion from NAD auxotrophy and thus that the overall selective advantage is best served when antibiotics are present when growing these strains.

The one exception to the small positive effect of antibiotics was when the R7AΔ*dct nadA*::pFUS2 strain was complemented by p994 and grown with a full complement of antibiotics, it then showed the lowest overall growth of any of the strains or the conditions examined. It was significantly lower than the same strain without antibiotics, which is the opposite trend seen in its p686 counterpart. This would strongly indicate that loss of the pFUS2 insert and subsequent reversion to NaMN prototrophy had a far higher advantage in this case than the maintenance of p994 i.e. it allowed for a higher production of NaMN (and subsequently NAD). This is an interesting finding in that it lends support to an argument that rather than secondary characteristics being the deciding factors as discussed above, it may in fact be due to deficiencies of the *nad* locus expression as conferred in p994. This could indicate that while the functional *nad* locus has indeed been captured in p994, some crucial upstream (or possibly downstream) regulatory function has been missed by the restriction fragment which may have some significant effect directly attributable to the regulation of NaMN production under starvation.

There are also several trivial explanations that could explain this, such as a mutation in thep994 fragment or interference from the vector itself, though none of these were investigated. A way to resolve this issue would be to examine directly the regulation of both the p686 *nad* genes and those of p994 to determine if their gross output was the same under starvation conditions.

4.4 Mechanisms behind NAD-mediated mutation

Investigations into the possible mechanisms involved in the mutator phenotype produced some slightly ambiguous results, but overall allowed for some informative conclusions. During the course of the two experiments that I undertook to investigate this area, I used strains that were deficient in two genes that are homologues for *mfd* and *recA* respectively. As both these genes are involved in different methods of DNA repair and have been implicated in other SIM systems in *E. coli* and *B. subtilis*(83, 86), their involvement or lack thereof may indicate what forms of DNA damage are occurring and how the mechanisms of SIM in *M. loti* compare with previously described systems in other prokaryotes.

The need for *recA* is well established in the Cairns system as previously described. It has the ability to bind ssDNA ends and recruit the DSBR complex to DSBs as well as its role in activating the SOS regulon through cleavage of the LexA repressor. Both these functions are essential to the error prone HR-DSBR model proposed in the Cairns system and the role it plays in DSBR are essential for the competing ADM model (12, 51).

On the other hand *mfd* is involved with a completely different form of DNA repair, being an essential component of transcription-coupled repair. During transcription, RNA polymerase (RNAP) can become stalled due to the presence of bulky lesions in the DNA, a lack of triphosphate nucleoside precursors or DNA-bound protein. For gene expression to continue, this stalled RNAP complex must either be disassociated from the DNA or assisted to proceed past the block and continue elongation. The role of Mfd is to bind to the stalled RNAP, and depending on the nature of the blockage, carry out dissociation or translocation. In specific relation to SIM it is the involvement of lesions in the DNA that are of relevance. When Mfd encounters an RNAP stalled due to a bulky lesion in the DNA, it binds both the RNAP and the upstream DNA in a nonspecific manner. Depending on the nature of the lesion and if NTP concentration is sufficient it can restart transcription through an ATP-driven translocation of the RNAP, pushing the RNA 3-end into the active site and allowing elongation to continue (76). Alternatively, to facilitate repair of the lesion which is now buried in the transcription bubble, Mfd will cause the disassociation of the RNAP complex. In conjunction with this Mfd also recruits UvrA that in turn recruits UvrB and UvrC, which together constitute the UvrABC nucleotide excision repair (NER) pathway, which then excise and repair the lesion (76, 89). Mutants deficient in Mfd do not show any significant changes

in mutation during exponential growth, but show significantly decreased levels of SIM in *B. subtilis* (72).

The first experiment involving the two strains, R7ANSΔ*mfd* and R7ANSΔ*recA*, and differing levels of exogenous nicotinic acid showed that the mutator phenotype still presented when nicotinic acid concentrations were high despite the loss of *recA* or *mfd*. There was no apparent change in the onset in the appearance of Dct^+ colonies in the deficient strains when compared to the wild type R7ANS, but they did show a slight consistent decrease in the total number of colonies forming. Considering the small numbers involved, it is possible that this discrepancy could be accounted for simply by variation, although both strains showed near identical divergence from the wild-type strain which would argue against this. The second experiment did not reveal such clear-cut results. Both *mfd* and *recA* deficient strains produced mutator colonies when coupled with either p994 or p686, but showed widely varying numbers depending on the plasmid used. Due to these inconsistencies and the fact that results from the R7ANSΔ*mfd*/p686 strain had to be disregarded due to contamination, it seems likely that there may have been issues in the procedures of this experiment that require repetitions to eliminate. However, the two experiments taken together indicate that the mutator phenotype was largely unaffected by the mutational inactivation of *mfd* or *recA*.

As the function of RecA is primarily concerned with the binding and repair of DSBs, it would appear that these do not form a significant proportion of the DNA damage leading to SIM in *M. loti*. That RecA, and by extension DSBs, are not required rules out both the HR-DSBR and ADM mechanisms that have been proposed for the Cairns system, suggesting that SIM in *M. loti* largely occurs through a different mechanism.

That two independent mechanism that have been found to be essential for SIM in other systems have at most small effects in *M. loti* would suggest either that SIM in this case arises through a novel mechanism or that the nature of the lesions made to the DNA are diverse and thus a large number of methods that can be employed to give rise to a mutation with no one mechanism being essential. That a diverse spectrum of lesions may be responsible lends support to the model that an increase in ROS-induced damage is the root cause.

4.5 Regulation of the *nad* locus

The regulation data produced strongly support the hypothesis that the production of NAD is increased during stationary phase. All of the genes monitoring the expression of the *nadABC* pathway showed an increase in expression at 72 hours and 144 hours when compared to cells that were still undergoing exponential growth (Figures 3.23-25). Though there was considerable variability, particularly during the exponential phase, the overall expression pattern was conserved across all three constructs. The dip in expression between 72 and 144 hours was also an interesting feature as it shows that while high expression was maintained, it is not necessary to maintain full expression for the mutator phenotype. This would indicate that once a high concentration of NAD(H) is achieved, expression of the *nad* genes can be reduced to maintain high concentrations of the respective enzymes, though after being kept at stationary phase for a further 72 hours, this drop could also reflect a loss of cell viability or a drop in overall metabolism. This observation also counters the argument that it is merely the buildup of the LacZ protein in the cell that is leading to an increase in expression at 72 hours. If this had been the case, then one would expect expression to steadily increase over the entire 144 hour period rather than show a slight decrease. The implication from this is that the synthesis of NaMN and by consequence NAD(P)(H) increases leading into stationary phase and is then maintained throughout stationary phase. This is counterintuitive to what one would expect to occur as cells are now in an arrested state and respiration would be limited. As one of the primary functions of NAD is to act as a hydride acceptor during respiration (88), it is unclear why cells would require such a dramatic increase in NAD production especially in comparison to exponentially-growing cells. This would indicate a novel function for NAD that requires the cofactor in high concentrations. Increases in concentration have been seen of both NAD and its phosphorylated form NADP in *S. cerevisiae,* which was primarily attributed to ameliorating the build-up of ROS during stationary phase (66-67). In *S. cerevisiae* though, NAD had a protective effect as seen by a lower mutation rate, which is the opposite of what was observed in this study. *S. cerevisiae* is a eukaryotic system, which means it has some properties not found in prokaryotic systems such as the enzyme PARP-1. This utilizes NAD as a sole substrate and is thought to be part of BER in eukaryotes and is important for genomic stability (42).

A possible explanation for increased NAD synthesis having the opposite effect in a prokaryotic system is that the increase in NAD changes the ratio of the NAD(H)/NADP(H)

pool in favor of NAD(H), which decreases the ability of the cell to reduce ROS as NADPH is the primary detoxifier. This is supported by a dramatic shift in the ratio of NADP and NAD in favour of NADP when *E. coli* cells are exposed to H_2O_2, indicating that a ratio in favour of NADP confers a protective state against oxidative stress (10). Thus if the cell can maintain a high concentration of pro-oxidant NAD(H) compared to anti-oxidant NADP(H) this may disrupt the protective state or even confer a mutagenic one. However, one could also speculate that even with an increase in NAD(H), the mechanisms that maintain NAD(H)/NADP(H) homeostasis may continue to maintain the existing ratio between the two, thus leading to an overall increase in NADP(H) levels. Further studies are required into the differences in both total concentrations and ratios of NAD(H) to NADP(H) within exponential cells as well as cells undergoing stationary phase, and how these differ in *M. loti* strains that either do or do not possess NAD biosynthesis pathways.

The regulation of the NAD biosynthesis genes is generally straight-forward, but there are still some perplexing aspects, particularly in regard to its relation to the mutator phenotype. The addition of higher concentrations of nicotinic acid to the medium affected the expression of the NAD biosynthesis genes. During exponential growth it led to inhibition of *nadABC* expression but the level of inhibition by the addition of nicotinic acid increased markedly as the cultures aged until by 144 hours expression of *nadABC* was negligible. The reason for this steady drop off may be that the NAD salvage pathway is used in preference over the biosynthetic pathway when high concentrations of NAD intermediate products are available. During exponential growth, NAD may be turned over at such a rate due to high levels of respiration associated with exponential growth that intracellular levels of NAD precursors may remain low enough that the *de novo* pathway is not completely repressed. As respiration and NAD consumption slows, intracellular concentrations become higher again as a result of the exogenous source and thus *de novo* synthesis is close to fully repressed. This is further supported by the fact that when no exogenous source of nicotinic acid was available, *de novo* expression was high (appears unregulated) but with addition of incremental concentrations of nicotinic acid expression initially dropped sharply followed by incremental decreases. This shows that the level of expression of the *de novo* pathway is relative the availability of substrates for and thus activity of the salvage pathway.

Evidence obtained from this study suggests that the *nadQ* gene is the regulator of the *de novo* pathway in *M. loti*. NAD biosynthesis is one of the best-characterized pathways in terms of

components and mechanisms, in both eukaryotes and prokaryotes. However despite this, information on the regulation of NAD biosynthesis is scarce (36). Until recently, the only gene identified as regulating *de novo* synthesis was the *nadR* gene which is a NAD-responsive repressor of *de novo* synthesis restricted to a cluster of enterobacteria (5, 27, 79). In several recent studies a number of new *de novo* regulatory genes have been uncovered, such as NiaR and NrtR which also map close to *denovo* synthesis genes and appear to have a repressive role mediated by metabolites of the salvage pathway. NiaR in particular shows strong affinity for nicotinic acid and is thought to regulate the import of nicotinic acid as well as *de novo* synthesis (78-79). A common feature of all the regulatory genes thus far described for the *de novo* synthesis pathway is that they appear to be fusions of metabolic genes and a helix-turn-helix DNA-binding motif. This function allows them to recognize particular cytosolic substrates, the binding of which alters their ability to recognize and bind specific sequences of DNA. In addition to this, the ability to undergo autoregulation appears to be an inherent property of many of these genes (78). The *nadQ* gene identified just upstream of the *nadABC* locus appears to share these characteristics, making it likely that it binds a substrate that allows it to repress the expression of the *de novo* pathway. This was shown with the Δ*nadQ nadB*::pFUS2 strain, where *nadB* expression was constitutive, approaching levels seen only when comparable strains were grown without any exogenous source of nicotinic acid. High expression levels were observed even when the strain was grown at a high concentration of nicotinic acid that fully repressed *nadABC* expression in $nadQ^{+}$ strains. This finding that exogenous nicotinic acid repressed *de novo* synthesis only when a intact copy of the *nadQ* gene was available strongly suggests that nicotinic acid is at least one of the substrates that binds NadQ to initiate repression.

Expression of *nadQ* itself appeared to be constitutive, as the expression from the *nadQ*-CMD fusion did not change in response to nicotinic acid concentration or growth phase. If cellular NadQ levels remain constant, then it would appear that substrate binding alters the DNA-binding affinity of NadQ to effect regulation of the *de novo* pathway.

It appears likely that nicotinic acid directly binds NadQ to cause repression rather than its direct downstream product of NaMN, which is also the direct product of the *nadABC* genes. This is evident from the partial repression of the mutator phenotype in NAD-prototrophic strains when high concentrations of nicotinic acid were added. This partial repression suggests that there is a discrepancy in NaMN output between the *de novo* pathway and the

salvage pathway (taking that the conversion of NaMN by NadE and NadD is common to both pathways). This suggests that the *de novo* pathway has a greater output of NaMN than the salvage pathway and that when the *de novo* pathway is repressed the salvage pathway is unable to meet the shortfall and thus the mutator phenotype is partially repressed. If NaMN were acting as the co-repressor, then it would be unlikely that repression of the mutator phenotype would also occur, as any discrepancy between the *de novo* and salvage pathways would lead to derepression of the *de novo* pathway. If one assumes that the primary signal binding occurs in the cytosol, as appears to be the common theme in *de novo* synthesis (78-79, 107), then repression only occurs once the co-repressor has entered the cell and therefore transport cannot be a limiting step. However, if intracellular nicotinic acid interacts with NadQ to repress *de novo* synthesis, and the reaction converting nicotinic acid into NaMN is the rate-limiting feature compared to *de novo* synthesis, this would explain the partial repression of the mutator phenotype in prototrophic strains. While it would seem that nicotinic acid is co-repressor, this does not exclude the possibility that several other salvage substrates such as the structurally similar nicotinamide may also bind NadQ with varying affinities and this could warrant further investigation. This would be consistent with previous literature, in which several substrates of the salvage pathway, including nicotinic acid, have been shown to repress the *de novo* pathway (79).

While this scenario would seem straightforward several issues remain that do not fit into a logical framework. This first and most important is that while it is clear that NadQ is a repressor, it also appeared to be essential for the mutator phenotype as p993 that contained *nadABC* but only a 3-prime portion of *nadQ* did not confer the mutator phenotype. This was puzzling as the effect of removing *nadQ* was uncontrolled and increased expression of the *de novo* pathway, which all other evidence indicated induced SIM. However, it has recently been shown that the Δ*nadQ* mutant still shows SIM (J. Sullivan, personal communication), throwing into doubt the interpretation that the lack of SIM induction by p993 was due to lack of *nadQ*. It seems possible that p993 may have an undetected mutation that results in the plasmid conferring only a low level of NAD synthesis. Alternatively, it is possible that the portion of *nadQ* remaining on p993 is somehow expressed and results in repression of *nadABC* expression that is not alleviated by the absence of the co-repressor. Clearly further work is required to resolve these issues.

A further question relates to how the activity of the *de novo* pathway is increased during stationary phase. From the runaway expression of the *de novo* pathway in the Δ*nadQ* strain and the inferred co-repression with nicotinic acid, it seems that it is derepression and not activation that results in the increase in *nadABC* expression. As the expression of *nadQ* is constitutive, this implies that there is a general decrease in the level of nicotinic acid and presumably other precursors of NAD salvage within the cell, which in turns points to either a pooling of NAD/NADP within the cell or an increase in the rapidity of turnover of NAD within the cell. While this is the most parsimonious explanation, it does not preclude a third unknown factor acting in an antagonistic manner to NadQ repression. Regardless, both models produce an end result whereby cells entering stationary phase need increased quantities of NAD leading to an increase in *de novo* synthesis when a source of salvage pathway precursors is not available.

The overall conclusions that can be drawn in regards to regulation of the NAD *de novo* synthesis pathway in *M. loti* are that expression is increased and maintained during stationary phase relative to exponential growth. Results suggest that NadQ bound to nicotinic acid functions as transcriptional repressor of the locus, though the binding of other salvage pathway precursors cannot be precluded.

4.6 Involvement of ROS scavenging

An increase in ROS both within and outside the cells has multiple effects in addition to DNA damage. ROS also damage both proteins and lipids through the Fenton reaction (93). Aside from general damage, ROS preferentially target enzymes containing iron-sulfur clusters in their active site, which leads both to a degradation in enzymatic activity and an increase in the levels of unincorporated iron (a driver of the Fenton reaction) (49, 113). This type of damage therefore also leads to an overall reduction of cell health and viability. To scavenge and eliminate ROS, cells utilize two basic classes of enzymes, superoxide dismutase (SOD) and catalase/peroxidase, which scavenge superoxide (O_2-) and hydrogen peroxide (H_2O_2) (49).

ROS scavenging could have multiple somewhat antagonistic effects in relation to SIM. On the one hand the efficient functioning of the ROS scavenging enzymes may help to

ameliorate the impacts of ROS on cell viability, allowing a greater number of cells to persist for longer in stationary phase and thus increasing both the population and time period from which mutants can arise. On the other hand, neutralizing or lowering the concentration of ROS would also decrease their resultant damage to DNA, thus limiting the potential pathway for the creation of mutants. Evidence from the literature tends to support the latter argument as deficiencies in SOD or catalase lead to increased rates of mutation in *P. putida* (52).

The reduction and delay of colony growth in the R7ANS/p994 strain may have resulted from the build-up of ROS negatively impacting cell growth. The ROS species hypothesized to be primarily responsible for this would be H_2O_2, as it is uncharged and can easily cross membranes, allowing it to be both excreted from cells and absorbed. This ability is exploited by both plants and other bacterial species to inhibit infecting and competing bacteria (49). Several lines of evidence lend credence to this argument. Firstly, inhibited colonies appeared at a time point similar to that seen with 'regular' Dct^+ colonies but the colonies were far smaller in size and took some days to reach the size of normal Dct^+ colonies. This may indicate that though these cells achieved the pre-requisite Dct^+ reversion, they were still being inhibited from their full growth potential by some other factor. Secondly, this inhibited phenotype appeared only on plates with a relatively high cell density, indicating that interactions with surrounding cells were at least partially responsible for this atypical mutator phenotype. This is unlikely to be due to nutritional deprivation as the cells in question are presumably Dct^+ and therefore have had their carbon source limitations removed. Therefore, inhibition from the byproducts of neighbouring cells such as H_2O_2 would be a likely candidate. Thirdly, this only occurred in R7ANS/p994, which carries the *nadQABC* locus. In contrast R7ANS/p686 did not show this inhibited growth. As p686 contains a significantly larger portion of the symbiosis island, it could be that one of the genes captured with it may have some relevance to the mutator phenotype such as ameliorating the negative effects associated with the build-up of ROS, both exogenously and endogenously. In particular a cytochrome C peroxidase gene *ccpR* (*msi380* (102)) is present on p686, under control of a NifA-regulated promoter. The *nifA* regulon is a well-characterized system that activates transcription under low oxygen levels, and is thus unlikely to be expressed in the growth conditions used (8, 99). However, it is conceivable that some low level of expression of this peroxidase did occur, possibly by read through from a vector promoter or through low-level basal expression, and that it may provide enough effect to relieve some of the effects evoked by ROS build-up.

The reasoning behind adding the catalase to the medium was that if the increase in nicotinic acid/NAD levels led to an increase in ROS, then adding a exogenous catalase may increase cell viability by breaking down H_2O_2 excreted by cells and thus decreasing the toxic inhibitory effect produced by dense numbers of cells under nutritional deprivation. In fact catalase did not seem to remove the inhibitory phenotype. This suggests that the build-up of exogenous H_2O_2 excreted by stressed cells was not responsible for the inhibitory phenotype. It could be argued that since the catalase was added exogenously then it is unlikely to affect ROS build-up within cells. However, this does not explain why the inhibition was cell density-dependent, as ROS build-up within cells should occur independent of the number of surrounding cells. It is also possible that the amount of catalase activity from the added enzyme was inadequate, though the fact that an effect was seen (albeit not the expected one) would argue against this. However, a host of other factors are released from cells under stress that could have inhibitory effects. Hence, though available evidence suggests that H_2O_2 is not the cause, a rich field of enquiry remains for other factors.

Despite not proving the initial hypothesis, the results did show that addition of catalase had an influence on the mutator phenotype. Exogenous catalase caused a decrease in Dct^+ colonies in both strains, which was consistent across all dilutions and therefore unlikely to be due to chance. There are several possible explanations for this decrease. The most likely explanation is that catalase decreased the exogenous levels of H_2O_2 (excreted from neighboring cells) leading to a decrease in the internal concentration of H_2O_2 and thus the mutation rate due decreased DNA damage. Catalases are some of the most efficient enzymes known, able to breakdown several million H_2O_2 molecules per second (14, 105). Thus, the vast majority of H_2O_2 excreted into the medium should be broken down. As the addition of catalase only diminished the number of Dct^+ colonies by a small amount, it is likely that H_2O_2 is rapidly converted to the damaging OH radical before the exogenous catalase can take effect. An extension of this reasoning is that this excreted extracellular H_2O_2 only contributes a small amount to the mutator phenotype. The added catalase, in lowering the extracellular concentration of H_2O_2, would allow cells producing excess H_2O_2 to lose more of it through diffusion across the cell membrane thus lowering the intracellular concentration of H_2O_2 and decreasing DNA damage/mutation rates. Either way this observation supports the idea that aspects of the mutator phenotype are cell density-dependent, as the rate in which H_2O_2 concentration increases in the medium would be based on the number of cells originally plated. This could account for observations such as the non-linear number of total colonies in

a dilution series, and the apparent delays seen in peak colony appearance, though it must be noted that no significant difference was noted in regards to either the time of initial colony appearance nor peak colony appearance when catalase was added.

Regardless, this experiment may suggest that accumulation of extracellular H_2O_2 exerts a positive influence on the mutator phenotype and is responsible at least in part for the production of Dct^+ mutants.

4.7 ROS model

A key question is how an increase in NAD synthesis during stationary phase in *M. loti* leads to the activation of the mutator phenotype. Even though NAD synthesis in yeast has previously been shown to be stimulated during stress conditions, it would initially seem an unlikely candidate as this has been primarily attributed to the protective effects afforded by high concentrations of NADPH (66-67). This of course lies in direct contradiction to what is needed for the mutator phenotype to occur which is essentially the formation of a highly mutagenic or genotoxic state which is in nature transitory. This suggests that in *M. loti*, NAD is being utilized in a novel way to support this state. Another major issue is the ubiquity of NAD derivatives as they are cofactors in over 300 reactions (5) which makes pinpointing which metabolic process is being affected somewhat difficult, though I will speculate herein which could be likely candidates.

The first possibility is the involvement NAD has in DNA ligation in prokaryotes. During ligation of single-strand breaks, NAD is consumed to adenylate the lysine residue in the active site of bacterial ligase to yield nicotinamide mononucleotide (NMN) thereby making NAD essential for aspects of DNA repair (36). It could be argued that DNA damage is likely to be greatly increased if the cells enter a mutagenic state, and that adequate repair mechanisms must be available for any mutation to become fixed (as the mutation arises through errors in repair, not from the repair itself *per se*). Also the process of ligation is necessary for the formation of NHEJ-induced SIM in *S. cerevisiae*, though this mechanism has yet to be linked to SIM in prokaryotes (43). As DNA ligation would be necessary for the formation of many mutations, an adequate supply of NAD to fuel this reaction is vital. There are two main issues that are counter to this hypothesis. Firstly, while the activity of

prokaryotic ligase may be increased during cells under genotoxic stress, levels of DNA damage would have to be untenably high in order to require levels of NAD over and above that required for cells undergoing exponential growth when respiration, the main consumer of NAD (36), is at its highest in order to fuel replication. Furthermore, this would not explain why either *de novo* synthesis or high exogenous levels of nicotinic acid appear to be vital for the mutator phenotype to occur. If an inadequate synthesis of NAD proved to be a limiting factor in the activity of ligation, then one would expect the primary result to be a loss of viability due to a buildup of deleterious lesions. This was not observed, but had it occurred it would have likely led to a reduction in the mutator phenotype rather than its complete absence (Dct^+ revertants were virtually nonexistent in wild-type R7ANS).

This leaves the possibility of an indirect effect of NAD, which seems most likely as an increase in NAD concentration will affect the fundamental state of the cell due to the use of NAD as a cofactor in such a wide range of reactions. In particular it is the role of NAD and its derivatives in redox reaction and the modulation of ROS that I have already touched upon briefly during this discussion that presents the most likely candidate for causing the mutator phenotype.

ROS are produced primarily as the byproduct of aerobic metabolism, which results mostly in superoxide (O_{2-}) and hydrogen peroxide (H_2O_2). Both these products are able to negatively affect cells by oxidizing iron-sulfur clusters present at the catalytic sites of a broad range of enzymes such as dehydratases and flavins (49, 113), resulting in a decrease in the efficient functioning of the cell. This also results in the release of free iron directly into the cytosol which has other implications that will be discussed at length. The other way in which these two ROS act is through the Fenton reaction. This reaction occurs through the oxidation of unincorporated ferrous iron that results in the formation of hydroxyl radicals (OH). Unincorporated ferrous iron localizes to all three classes of biomolecules, which, due to the extreme reactivity of the OH, occurs at near the diffusion rate, thus resulting in localized damage. It is this end result of ROS that is so damaging to cells and the damage to DNA is primary cause of genotoxic/mutagenic effects associated with ROS (49, 93).

Damage caused by ROS may also be able to elevate mutation rates by damage to proteins involved in DNA replication, specifically the fidelity of DNA polymerases, and through damage to transcription/translation machinery that again can lead to changes in replicative fidelity downstream (106). Oxidative damage causes a wide range of potentially mutagenic

damage to DNA and its precursors. It can cause single- and double-strand breaks in DNA by interacting with either the phosphate or deoxyribose in the phosphodiester backbone of DNA, as well as directly oxidizing the base residues in DNA, leading to base changes and dimer formation (93). Oxidation of the dNTP pool can also occur, producing 8-oxo-dGTP in particular which is frequently incorporated to base pair with adenine, providing a fertile source of mutation (35, 48). This diverse range of lesions shows some consistency with the results obtained from the strains with deletions of genes in particular DNA repair pathways described herein, where the mutator phenotype was only reduced by a small amount indicating that no single type of lesion was primarily responsible for the appearance of Dct^+ revertants

Aside from the ability to cause such a large array of lesions in DNA, there is also an emerging body of evidence that suggests that ROS are the major cause of mutagenesis *in vivo*, particularly under stress conditions. Several studies have shown that development of antibiotic resistance may be intrinsically linked to oxidative stress being caused by the drug's mode of action (22).

A variety of studies have also shown that removal of key genes that scavenge ROS produce strains that mutate at a constitutively high rate. For example bacteria lacking certain stationary phase catalases and SOD (106) have been shown have high mutation rates, and that this state can be induced by high concentration of exogenous H_2O_2 (44). In relation to this, mutant strains of the GO system that cleans the nucleotide pool of aberrant nucleotides have been shown to be in a mutator state which is further exacerbated by the addition of exogenous H_2O_2 (110).

Increases in oxidative stress would appear to be a likely candidate responsible for the mutator phenotype; the question then is how does an increase in NAD biosynthesis directly lead to an increase in ROS? The first issue is whether NAD directly causes the phenotype by somehow generating ROS, or is the mechanism somewhat less direct in that it facilitates the damage caused by a preexisting high level of ROS i.e. is it merely exacerbating a physiological state that is occurring independently?

As far as an increase in NAD directly leading to a corresponding increase in ROS, there is a general dearth of evidence in the literature to support this. As suggested earlier, an increase in ROS could result from a change in the ratio between NAD(H) and NADP(H). As

NADP/NADPH is primarily responsible for the scavenging of ROS such as through NADP-dependent catalases (14), reduction in its concentration could easily lead to increase in ROS. However, several issues are left unexplained in this scenario as to why would a change in ratio happen as presumably enzymatic pathways synthesizing NADP are still functioning and also where is the NAD being used/sequestered to? Data on the regulation of the *de novo* pathway from this study suggests that the *de novo* pathway is largely switched on by a lack of repression as a result of lower concentrations of NAD intermediates. This would indicate that NAD is being utilized or sequestered at a higher rate than it is broken down, and for a considerable amount of time.

The second possibility of NAD exacerbating the effects of ROS seems more likely. There are several studies that show an increase in the levels of ROS in cells that are either stressed or non-growing (50). If the assumption is made that there is a buildup of ROS in *M. loti* cells under starvation and that it is a typical physiological response, independent of NAD, then several mechanisms present themselves. Firstly, again the diminished contribution of NADP could be a factor, though in this case it is the lack of ability to rid the cell of already high ROS levels rather than contributing to their generation that is considered. Secondly, is the potential of NADH to act as the reducing agent in the cycling of iron from Fe^{3+} back to Fe^{2+}. This role of NAD has been suggested as a lead cause of mutagenesis in *E. coli* exposed to high concentrations of exogenous hydrogen peroxide and NAD has been established to have an affinity for reducing Fe^{3+} sixteen times higher than NADP (10). This allows for a very efficient turnover of Fe^{2+} which in turn resupplies iron for the Fenton reaction to take place. As stated, the Fenton reaction is the primary cause of oxidative damage to DNA through the creation of a hydroxyl radical. With this constant recycling of iron, mediated by high levels of NADH, a highly mutagenic state could be induced even without necessarily a huge increase in ROS levels. In relation to this, H_2O_2 and O_{2-} cause the release of enzyme-associated iron, presumably causing an increase in free iron within the cell. The increase in free iron concentration and its subsequent consumption of NADH during its reduction cycling in the Fenton reaction would both contribute to higher rates of mutagenesis as well as account for a need for increased synthesis of NAD. While this model would certainly support many of the findings of this work such as the effect of catalase, diversity of DNA lesions and up-regulation of NAD biosynthesis, there is not as yet any direct evidence to support this.

Several other lines of evidence must first be gathered to test the models stated herein. Firstly, examining the ratio of NAD(H) to NADP(H) within both exponential and stationary phase cells, as well as their actual cellular concentrations, would reveal much about the mechanisms being induced. Also it would be useful to establish a more detailed picture of the regulation of the NAD *de novo* synthesis pathway, in particular what other NAD breakdown products such as nicotinamide, NaMN, nicotinic acid riboside and nicotinamide riboside (36) may also be involved in the repression of the *de novo* pathway. The roles of the NAD salvage pathway would also be of interest, particularly those concerned with the transport and conversion of NA to NaMN and whether these are also regulated either by *nadQ* or at least show a similar phase-specific up-regulation. To this end, establishing the regulation of *nadD* and *nadE* would be useful as they are common to both the *de novo* pathway and the salvage pathway and present a limiting step in both reactions. They are generally assumed to be expressed constitutively (79) but up-regulation is not impossible as in some systems they are clustered with putative *nad* regulatory genes (78). Coupling of either of these genes either to an inducible or low-yield promoter would also help rule out the possibility of any role played by the buildup of NaMN rather than NAD itself, which though seen as a more remote possibility has not been definitively ruled out.

It would also be useful to establish whether or not levels of ROS are significantly increased during stationary phase, though there is a significant body of literature that supports this. In this same vein, the levels of free iron under stationary and exponential phases should be examined as well as whether steps to lower these such as a chelating agent lower the rates of stationary phase mutagenesis. Thirdly, further examination of the role of both genes involved in DNA repair and scavenging ROS could garner greater insight. Manipulations of BER, NER and MMR pathways would prove useful as well as manipulations of the homologues of error-prone DNA polymerases. The GO system, which operates to purify the cells dNTP pool of oxidized and aberrant dNTPs (including those incorporated into DNA), would also be of interest as it is amenable to direct interference by ROS and lies upstream of most of the DNA repair pathways I have mentioned (110). The roles of catalase, peroxidase and SOD enzymes also present viable targets in untangling the possible effects of ROS. Recent work carried out in the lab has shown that catalase-deficient strains show a remarkably high increase in mutation rates during stationary phase, which lends additional credence towards this line of investigation (Shaun Ferguson, personal communication).

One particular experimental setup that is absent from this thesis is a basic reconstruction test. A reconstruction test is where a small number of revertants are added to a culture of non-revertant cells, to examine how long it takes for the revertant colonies to appear. If the revertants take an extended period of time to appear, it may be that they were pre-existing in the original culture and not induced by stress. However work has been carried out in this area by others using some but not all of the strains relevant to this particular (6) and found that the revertant colonies appeared at a uniform time and not in a staggered manner. Hence this issue can be viewed as resolved. Also it would appear even from my own work that R7A/R7ANS strains display low or no reversion to Dct^+ during exponential growth, as can be seen from virtually no Dct^+ colonies appearing when R7ANS is grown on media containing relatively low concentrations of nicotinic acid (despite clearly having the genetic potential to form revertants) which alone indicates that Dct^+ revertants from exponential growth only form a statistically negligible portion of Dct^+ revertants.

The findings described in this thesis are of considerable significance. While this study has only examined a small part of the possible mechanisms in the *M. loti* mutator phenotype in practical terms, it does confirm a novel and possibly critical phenomenon. As the regulation of NAD synthesis is poorly understood the extent to which this novel mechanism is distributed among other taxa is currently unknown. However, regulatory genes with similarity to *nadQ* are found in many bacteria including pathogenic mycobacteria. Interestingly, it has recently been shown that *Mycobacterium tuberculosis* undergoes high rates of mutation due to oxidative stress in non-growing cells during latency in the host (92).

While it would seem that mechanisms that allow for transient hypermutation are widespread, they are also incredibly diverse in their modes of action, indicating that they have evolved independently multiple times. However, virtually all genes that have previously been described to be involved with SIM in other systems (with the exception of the competence factors ComK and ComA in *B. subtilis*(103)) have been involved directly with DNA repair and/or processing, or lead directly to the activation of genes involved with DNA transactions, thus providing an obvious link between the gene concerned and the mechanism for mutation. As far as I can ascertain, this is the first time that key metabolic genes have been identified as involved or responsible for SIM. Furthermore, it is one of the few instances in the literature where addition of a locus has led directly to formation of a SIM state in the receiving strain. This particular mechanism may present a new strategy amongst a varied repertoire available

to prokaryotes. More importantly it represents a unique drug target. As antibiotic resistance has become a major problem, it has become clear that variable rates of mutation amongst pathogenic bacteria has become a major driver of this, and that the state induced by the antibiotics themselves could either trigger a hypermutable state or create an artificial one (22, 53, 55). Thus developing drug targets that are able to work in conjunction with antibiotics to suppress the formation of resistance presents a viable strategy. In this case drugs that targeted the ability of *nadE* or *nadD* analogues would be prime targets as they are shared in common and are essential to both the *de novo* and salvage pathways of NAD synthesis, thus suppression of their activity would essentially abrogate any synthesis of NAD. This may not only lower the level of mutagenesis occurring in pathogenic bacteria undergoing oxidative stress but would also be expected to severely limit growth by eliminating the availability of an essential cofactor. Drug targeting of NAD and derivative pathways is not a new concept as disabling the production of NADP has previously been suggested (5), as it proved to be a key metabolic junction from *in silico* analysis. However, from extrapolations of the present study such a move may actually prove to be detrimental as it could create a similar cellular environment as is created in mutator *M. loti*. Thus further investigation would also help to refine the most beneficial targets for drug action in this pathway.

These results show further significance in that they highlight possibly new roles associated with NAD homeostasis, whose functions are otherwise very well characterized. They also shed new light on the regulation of NAD in this particular organism which may have obvious implications across diverse taxa as it shows the utilization of a cellular process, that has always been regarded to serve a protective function, to in fact operate inversely to this. This has further added to unraveling the mutator phenotype puzzle, a conundrum that has arguably still not been resolved since Cairns put forward his 1988 paper.

In conclusion, this study has shown that there is a clear mutator phenotype present in the *M. loti* strain R7A, and that this is conferred by the presence of a set of NAD *de novo* biosynthesis genes. These genes show increased expression during stationary phase and are repressed by the presence of increased concentrations of exogenous nicotinic acid. The *nadQ* gene, which lies just upstream of the *nadABC* locus, might bind nicotinic acid to initiate transcriptional repression of *nadABC*. Examination of the effects of both transcriptional repair and double strand break repair mediated mutagenesis showed that they only exhibited a minor effect and are not significant parts of SIM in *M. loti*. Addition of exogenous catalase

was also seen to have only minor effects on the mutator phenotype and therefore build up extracellular H_2O_2 is not a primary driver of SIM. Finally, I propose a model in which SIM is caused by an increase in DNA damage during stationary phase by ROS, which an increase in NAD helps to promote.

5. References

1. **Andersson, D. I., and D. Hughes.** 2009. Gene amplification and adaptive evolution in bacteria. Annual Review of Genetics **43:**167-195.

2. **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.

3. **Antoine, R., S. Alonso, D. Raze, L. Coutte, S. Lesjean, E. Willery, C. Locht, and F. Jacob-Dubuisson.** 2000. New virulence-activated and virulence-repressed genes identified by systematic gene inactivation and generation of transcriptional fusions in *Bordetella pertussis*. Journal of Bacteriology **182:**5902-5905.

4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. Journal of General Microbiology **84:**188-198.

5. **Bi, J., H. Wang, and J. Xie.** 2011. Comparative genomics of NAD(P) biosynthesis and novel antibiotic drug targets. Journal of Cellular Physiology **226:**331-40.

6. **Bjedov, I., O. Tenaillon, B. Gerard, V. Souza, E. Denamur, M. Radman, F. Taddei, and I. Matic.** 2003. Stress-induced mutagenesis in bacteria. Science **300:**1404-9.

7. **Bjorkman, J., and D. I. Andersson.** 2000. The cost of antibiotic resistance from a bacterial perspective. Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy **3:**237-245.

8. **Bobik, C., E. Meilhoc, and J. Batut.** 2006. FixJ: a major regulator of the oxygen limitation response and late symbiotic functions of *Sinorhizobium meliloti*. Journal of Bacteriology **188:**4890-4902.

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

10. **Brumaghim, J. L., Y. Li, E. Henle, and S. Linn.** 2003. Effects of hydrogen peroxide upon nicotinamide nucleotide metabolism in *Escherichia coli*: changes in enzyme levels and nicotinamide nucleotide pools and studies of the oxidation of NAD(P)H by Fe(III). Journal of Biological Chemistry **278:**42495-504.

11. **Cairns, J.** 1998. Mutation and cancer: The antecedents to our studies of adaptive mutation. Genetics **148:**1433-1440.

12. **Cairns, J., and P. L. Foster.** 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics **128:**695-701.

13. **Cairns, J., J. Overbaugh, and S. Miller.** 1988. The origin of mutants. Nature **335:**142-145.

14. **Chelikani, P., I. Fita, and P. C. Loewen.** 2004. Diversity of structures and properties among catalases. Cellular and Molecular Life Sciences **61:**192-208.

15. **Cohen, S. E., C. A. Lewis, R. A. Mooney, M. A. Kohanski, J. J. Collins, R. Landick, and G. C. Walker.** 2010. Roles for the transcription elongation factor

NusA in both DNA repair and damage tolerance pathways in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America **107:**15517-15522.

16. **Debora, B. N., L. E. Vidales, R. Ramirez, M. Ramirez, E. A. Robleto, R. E. Yasbin, and M. Pedraza-Reyes.** 2011. Mismatch repair modulation of MutY activity drives *Bacillus subtilis* stationary-phase mutagenesis. Journal of Bacteriology **193:**236-245.

17. **Dombrecht, B., J. Vanderleyden, and J. Michiels.** 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.

18. **Dong, T., and H. E. Schellhorn.** 2009. Control of RpoS in global gene expression of *Escherichia coli* in minimal media. Molecular Genetics and Genomics **281:**19-33.

19. **Drake, J. W.** 1991. A constant rate of spontaneous mutation in DNA-based microbes. Proceedings of the National Academy of Sciences of the United States of America **88:**7160-7164.

20. **Drake, J. W., B. Charlesworth, D. Charlesworth, and J. F. Crow.** 1998. Rates of spontaneous mutation. Genetics **148:**1667-1686.

21. **Dudas, A., and M. Chovanec.** 2004. DNA double-strand break repair by homologous recombination. Mutation research **566:**131-67.

22. **Dwyer, D. J., M. A. Kohanski, and J. J. Collins.** 2009. Role of reactive oxygen species in antibiotic action and resistance. Current Opinion in Microbiology **12:**482-9.

23. **Eckert, B., and C. F. Beck.** 1989. Overproduction of transposon TN10-encoded tetracycline resistance protein results in cell-death and loss of membrane-potential. Journal of Bacteriology **171:**3557-3559.

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

25. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans* Proceedings of the National Academy of Sciences of the United States of America **76:**1648-1652.

26. **Flowers, R. S., S. E. Martin, D. G. Brewer, and Z. J. Ordal.** 1977. Catalase and enumeration of stressed *Staphylococcus aureus* cells. Applied and Environmental Microbiology **33:**1112-1117.

27. **Foster, J. W., Y. K. Park, T. Penfound, T. Fenger, and M. P. Spector.** 1990. Regulation of NAD metabolism in *Salmonella typhimurium* - Molecular sequence analysis of the bifunctional NadR regulator and the *nadA-pnuC* operon. Journal of Bacteriology **172:**4187-4196.

28. **Foster, P. L.** 2007. Stress-induced mutagenesis in bacteria. Critical Reviews in Biochemistry and Molecular Biology **42:**373-397.

29. **Foster, P. L., and J. Cairns.** 1992. Mechanisms of directed mutation. Genetics **131:**783-789.

30. **Foster, P. L., and W. A. Rosche.** 1999. Adaptive mutation in *Escherichia coli* strain FC40. Journal of Genetics **78:**7-11.

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

32. **Frisch, R. L., Y. Su, P. C. Thornton, J. L. Gibson, S. M. Rosenberg, and P. J. Hastings.** 2010. Separate DNA Pol II- and Pol IV-dependent pathways of stress-induced mutation during double-strand-break repair in *Escherichia coli* are controlled by RpoS. Journal of Bacteriology **192:**4694-4700.

33. **Galhardo, R. S., R. Do, M. Yamada, E. C. Friedberg, P. J. Hastings, T. Nohmi, and S. M. Rosenberg.** 2009. DinB upregulation is the sole role of the SOS response in stress-induced mutagenesis in *Escherichia coli*. Genetics **182:**55-68.

34. **Galhardo, R. S., P. J. Hastings, and S. M. Rosenberg.** 2007. Mutation as a stress response and the regulation of evolvability. Critical Reviews in Biochemistry and Molecular Biology **42:**399-435.

35. **Gawel, D., M. D. Hamilton, and R. M. Schaaper.** 2008. A novel mutator of *Escherichia coli* carrying a defect in the *dgt* gene, encoding a dGTP triphosphohydrolase. Journal of Bacteriology **190:**6931-9.

36. **Gazzaniga, F., R. Stebbins, S. Z. Chang, M. A. McPeek, and C. Brenner.** 2009. Microbial NAD metabolism: lessons from comparative genomics. Microbiology and Molecular Biology Reviews **73:**529-41.

37. **Gentile, C. F., S. C. Yu, S. A. Serrano, P. J. Gerrish, and P. D. Sniegowski.** 2011. Competition between high- and higher-mutating strains of *Escherichia coli*. Biology Letters **7:**422-424.

38. **Gibson, J. L., M. J. Lombardo, P. C. Thornton, K. H. Hu, R. S. Galhardo, B. Beadle, A. Habib, D. B. Magner, L. S. Frost, C. Herman, P. J. Hastings, and S. M. Rosenberg.** 2010. The sigma E stress response is required for stress-induced mutation and amplification in *Escherichia coli*. Molecular Microbiology **77:**415-430.

39. **Giraud, A., I. Matic, O. Tenaillon, A. Clara, M. Radman, M. Fons, and F. Taddei.** 2001. Costs and benefits of high mutation rates: Adaptive evolution of bacteria in the mouse gut. Science **291:**2606-2608.

40. **Gonzalez, C., L. Hadany, R. G. Ponder, M. Price, P. J. Hastings, and S. M. Rosenberg.** 2008. Mutability and importance of a hypermutable cell subpopulation that produces stress-induced mutants in *Escherichia coli*. Plos Genetics **4**.

41. **Gupta, R., D. Barkan, G. Redelman-Sidi, S. Shuman, and M. S. Glickman.** 2011. Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. Molecular Microbiology **79:**316-330.

42. **Hageman, G. J., and R. H. Stierum.** 2001. Niacin, poly(ADP-ribose) polymerase-1 and genomic stability. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis **475:**45-56.

43. **Halas, A., H. Baranowska, A. Podlaska, and E. Sledziewska-Gojska.** 2009. Evaluation of the roles of Pol zeta and NHEJ in starvation-associated spontaneous mutagenesis in the yeast *Saccharomyces cerevisiae*. Current Genetics **55:**245-51.

44. **Hanyu, M., H. Fujimoto, K. Tejima, and K. Saeki.** 2009. Functional differences of two distinct catalases in *Mesorhizobium loti* MAFF303099 under free-living and symbiotic conditions. Journal of Bacteriology **191:**1463-1471.

45. **Heidenreich, E.** 2007. Adaptive mutation in *Saccharomyces cerevisiae*. Critical Reviews in Biochemistry and Molecular Biology **42:**285-311.

46. **Heidenreich, E., H. Eisler, T. Lengheimer, P. Dorninger, and F. Steinboeck.** 2010. A mutation-promotive role of nucleotide excision repair in cell cycle-arrested cell populations following UV irradiation. DNA Repair **9:**96-100.

47. **Hendrickson, H., E. S. Slechta, U. Bergthorsson, D. I. Andersson, and J. R. Roth.** 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 of the United States of America **99:**2164-2169.

48. **Hori, M., C. Ishiguro, T. Suzuki, N. Nakagawa, T. Nunoshiba, S. Kuramitsu, K. Yamamoto, H. Kasai, H. Harashima, and H. Kamiya.** 2007. UvrA and UvrB enhance mutations induced by oxidized deoxyribonucleotides. DNA Repair **6:**1786-93.

49. **Imlay, J. A.** 2008. Cellular defenses against superoxide and hydrogen peroxide. Annual Review of Biochemistry **77:**755-776.

50. **Jakubowski, W., T. Bilinski, and G. Bartosz.** 2000. Oxidative stress during aging of stationary cultures of the yeast *Saccharomyces cerevisiae*. Free Radical Biology and Medicine **28:**659-664.

51. **Janion, C.** 2008. Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. International Journal of Biological Sciences **4:**338-344.

52. **Jatsenko, T., A. Tover, R. Tegova, and M. Kivisaar.** 2010. Molecular characterization of Rif(r) mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. Mutation Research **683:**106-14.

53. **Jayaraman, R.** 2010. Novel mechanisms of emergence of multidrug resistance/tolerance. Current Science **99:**1008-1010.

54. **Kofoid, E., U. Bergthorsson, E. S. Slechta, and J. R. Roth.** 2003. Formation of an F ' plasmid by recombination between imperfectly repeated chromosomal rep sequences: a closer look at an old friend (F '(128) pro lac). Journal of Bacteriology **185:**660-663.

55. **Kohanski, M. A., M. A. DePristo, and J. J. Collins.** 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Molecular Cell **37:**311-20.

56. **Ksiazek, K.** 2010. Bacterial aging: from mechanistic basis to evolutionary perspective. Cellular and Molecular Life Sciences **67:**3131-3137.

57. **Lightfield, J., N. R. Fram, and B. Ely.** 2011. Across bacterial phyla, distantly-related genomes with similar genomic GC content have similar patterns of amino acid usage. Plos One **6:**e17677

58. **Liu, G., J. Foster, P. Manlapazramos, and B. M. Olivera.** 1982. Nucleotide salvage pathway for NAD biosynthesis in *Salmonella typhimurium*. Journal of Bacteriology **152:**1111-1116.

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

60. **Lombardo, M. J., I. Aponyi, and S. M. Rosenberg.** 2004. General stress response regulator RpoS in adaptive mutation and amplification in *Escherichia coli*. Genetics **166:**669-680.

61. **Luria, S. E., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28:**491-511.

62. **Masson-Boivin, C., E. Giraud, X. Perret, and J. Batut.** 2009. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends in Microbiology **17:**458-66.

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

64. **Miller, J. H.** 2005. Perspective on mutagenesis and repair: The standard model and alternate modes of mutagenesis. Critical Reviews in Biochemistry and Molecular Biology **40:**155-179.

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

66. **Minard, K. I., and L. McAlister-Henn.** 2010. Pnc1p supports increases in cellular NAD(H) levels in response to internal or external oxidative stress. Biochemistry **49:**6299-301.

67. **Minard, K. I., and L. McAlister-Henn.** 2009. Redox responses in yeast to acetate as the carbon source. Archives of Biochemistry and Biophysics **483:**136-43.

68. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.

69. **Pitcher, R. S., N. C. Brissett, and A. Doherty.** 2007. Nonhomologous end joining in bacteria: A microbial perspective. Annual Review of Microbiology **61:**259-282.

70. **Ponder, R. G., N. C. Fonville, and S. M. Rosenberg.** 2005. A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation. Molecular Cell **19:**791-804.

71. **Powell, S. C., and R. M. Wartell.** 2001. Different characteristics distinguish early versus late arising adaptive mutations in *Escherichia coli* FC40. Mutation Research **473:**219-228.

72. **Pybus, C., M. Pedraza-Reyes, C. A. Ross, H. Martin, K. Ona, R. E. Yasbin, and E. Robleto.** 2010. Transcription-associated mutation in *Bacillus subtilis* cells under stress. Journal of Bacteriology **192:**3321-8.

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

74. **Ramsay, J. P., J. T. Sullivan, N. Jambari, C. A. Ortori, S. Heeb, P. Williams, D. A. Barrett, I. L. Lamont, and C. W. Ronson.** 2009. A LuxRI-family regulatory

system controls excision and transfer of the *Mesorhizobium loti* strain R7A symbiosis island by activating expression of two conserved hypothetical genes. Molecular Microbiology **73:**1141-1155.

75. **Ramsay, J. P., J. T. Sullivan, G. S. Stuart, I. L. Lamont, and C. W. Ronson.** 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.

76. **Roberts, J., and J. S. Park.** 2004. Mfd, the bacterial transcription repair coupling factor: translocation, repair and termination. Current Opinion in Microbiology **7:**120-5.

77. **Robleto, E. A., R. Yasbin, C. Ross, and M. Pedraza-Reyes.** 2007. Stationary phase mutagenesis in *B.subtilis*: A paradigm to study genetic diversity programs in cells under stress. Critical Reviews in Biochemistry and Molecular Biology **42:**327-339.

78. **Rodionov, D. A., J. De Ingeniis, C. Mancini, F. Cimadamore, H. Zhang, A. L. Osterman, and N. Raffaelli.** 2008. Transcriptional regulation of NAD metabolism in bacteria: NrtR family of Nudix-related regulators. Nucleic Acids Research **36:**2047-59.

79. **Rodionov, D. A., X. Li, I. A. Rodionova, C. Yang, L. Sorci, E. Dervyn, D. Martynowski, H. Zhang, M. S. Gelfand, and A. L. Osterman.** 2008. Transcriptional regulation of NAD metabolism in bacteria: genomic reconstruction of NiaR (YrxA) regulon. Nucleic Acids Research **36:**2032-46.

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

81. **Ronson, C. W., and S. B. Primrose.** 1979. Carbohydrate-metabolism in *Rhizobium trifolii* - Identification and symbiotic properties of mutants. Journal of General Microbiology **112:**77-88.

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

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

84. **Roth, J. R.** 2010. Genetic adaptation: A new piece for a very old puzzle. Current Biology **20:**R15-R17.

85. **Roth, J. R., E. Kofoid, F. P. Roth, O. G. Berg, J. Seger, and D. I. Andersson.** 2003. Regulating general mutation rates: Examination of the hypermutable state model for Cairnsian adaptive mutation. Genetics **163:**1483-1496.

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

87. **Ryan, F. J.** 1955. Spontaneous mutation in non-dividing bacteria. Genetics **40:**726-738.

88. **Sauve, A. A.** 2008. NAD+ and vitamin B3: from metabolism to therapies. The Journal of Pharmacology and Experimental Therapeutics **324:**883-93.

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

90. **Schlacher, K., and M. F. Goodman.** 2007. Timeline - Lessons from 50 years of SOS DNA-damage-induced mutagenesis. Nature Reviews Molecular Cell Biology **8:**587-594.

91. **Sheng, Y., V. Mancino, and B. Birren.** 1995. Transformation of *Escherichia coli* with large DNA-molecules by electroporation. Nucleic Acids Research **23:**1990-1996.

92. **Sherman, D. R., and S. Gagneux.** 2011. Estimating the mutation rate of *Mycobacterium tuberculosis* during infection. Nature Genetics **43:**400-401.

93. **Sigler, K., J. Chaloupka, J. Brozmanova, N. Stadler, and M. Hofer.** 1999. Oxidative stress in microorganisms - I - Microbial vs. higher cells - Damage and defenses in relation to cell aging and death. Folia Microbiologica **44:**587-624.

94. **Slechta, E., K. L. Bunny, S. E. Gerum, E. C. Kofoid, E. Kugelberg, D. I. Andersson, and J. R. Roth.** 2003. Mutagenesis in the Cairns system requires co-amplification of *dinB*+ (error prone polymerase) with the selected *lac* allele. Abstracts of the General Meeting of the American Society for Microbiology **103:**H-100.

95. **Slechta, E. S., K. L. Bunny, E. Kugelberg, E. Kofoid, D. I. Andersson, and J. R. Roth.** 2003. Adaptive mutation: General mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac*. Proceedings of the National Academy of Sciences of the United States of America **100:**12847-12852.

96. **Slechta, E. S., J. Harold, D. I. Andersson, and J. R. Roth.** 2002. The effect of genomic position on reversion of a *lac* frameshift mutation (lacIZ33) during non-lethal selection (adaptive mutation). Molecular Microbiology **44:**1017-1032.

97. **Sniegowski, P. D., P. J. Gerrish, and R. E. Lenski.** 1997. Evolution of high mutation rates in experimental populations of *E. coli*. Nature **387:**703-705.

98. **Stumpf, J. D., A. R. Poteete, and P. L. Foster.** 2007. Amplification of *lac* cannot account for adaptive mutation to Lac+ in *Escherichia coli*. Journal of Bacteriology **189:**2291-9.

99. **Sullivan, J. T., S. D. Brown, R. R. Yocum, and C. W. Ronson.** 2001. The bio operon on the acquired symbiosis island of *Mesorhizobium* sp strain R7A includes a novel gene involved in pimeloyl-CoA synthesis. Microbiology-Sgm **147:**1315-1322.

100. **Sullivan, J. T., B. D. Eardly, P. VanBerkum, and C. W. Ronson.** 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Applied and Environmental Microbiology **62:**2818-2825.

101. **Sullivan, J. T., H. N. Patrick, W. L. Lowther, D. B. Scott, and C. W. Ronson.** 1995. Nodulating strains of *Rhizobium Loti* arise through chromosomal symbiotic gene-transfer in the environment. Proceedings of the National Academy of Sciences of the United States of America **92:**8985-8989.

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

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

104. **Sung, H. M., G. Yeamans, C. A. Ross, and R. E. Yasbin.** 2003. Roles of YqjH and YqjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA

polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*. Journal of Bacteriology **185:**2153-2160.

105. **Switala, J., and P. C. Loewen.** 2002. Diversity of properties among catalases. Archives of Biochemistry and Biophysics **401:**145-154.

106. **Tarassova, K., R. Tegova, A. Tover, R. Teras, M. Tark, S. Saumaa, and M. Kivisaar.** 2009. Elevated mutation frequency in surviving populations of carbon-starved *rpoS*-deficient *Pseudomonas putida* is caused by reduced expression of superoxide dismutase and catalase. Journal of Bacteriology **191:**3604-14.

107. **Teramoto, H., M. Suda, M. Inui, and H. Yukawa.** 2010. Regulation of the expression of genes involved in NAD de novo biosynthesis in *Corynebacterium glutamicum*. Applied and Environmental Microbiology **76:**5488-95.

108. **Treangen, T. J., A. L. Abraham, M. Touchon, and E. P. C. Rocha.** 2009. Genesis, effects and fates of repeats in prokaryotic genomes. FEMS Microbiology Reviews **33:**539-571.

109. **Vanbleu, E., K. Marchal, and J. Vanderleyden.** 2004. Genetic and physical map of the pLAFR1 vector. DNA Sequence **15:**225-227.

110. **Vidales, L. E., L. C. Cardenas, E. Robleto, R. E. Yasbin, and M. Pedraza-Reyes.** 2009. Defects in the error prevention oxidized guanine system potentiate stationary-phase mutagenesis in *Bacillus subtilis*. Journal of Bacteriology **191:**506-13.

111. **Walsh, J. M., L. A. Hawver, and P. J. Beuning.** 2011. *Escherichia coli* Y family DNA polymerases. Frontiers in Bioscience **16:**3164-3182.

112. **Weaver, J. E.** 2003. Cryptic *dct* genes of Mesorhizobia. PhD. University of Otago, Dunedin, New Zealand.

113. **Woodmansee, A. N., and J. A. Imlay.** 2002. Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. Journal of Biological Chemistry **277:**34055-34066.

114. **Wozniak, R. A. F., and M. K. Waldor.** 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nature Reviews Microbiology **8:**552-563.

115. **Wrande, M., J. R. Roth, and D. Hughes.** 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 of the United States of America **105:**11863-8.

116. **Yeiser, B., E. D. Pepper, M. F. Goodman, and S. E. Finkel.** 2002. SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. Proceedings of the National Academy of Sciences of the United States of America **99:**8737-8741.

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