GENETIC STUDIES ON THE NITROGEN-FIXING BACTERIUM

RHIZOBIUM TRIFOLII

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I hereby declare that this thesis has been composed by myself, and that all the work herein is my own.
Abstract

The efficiency of a variety of common mutagens in producing mutation in *R. trifolii* P3, as exemplified by the induction of rifampicin-resistant mutants, was examined. Ethyl methanesulphonate, methyl methanesulphonate, decarbamoyl mitomycin C, nitrous acid and gamma rays did not mutate *R. trifolii* P3. Two other mutagens, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and ultraviolet light (UV) produced a notable increase over the spontaneous mutation frequency, the former being the most effective. The maximum induced frequency obtained, \(5.5 \times 10^{-6}\), although 400 times the spontaneous frequency, was considered to be too low for the isolation of specific non-selectable mutants.

Attempts to increase the susceptibility of this species to mutation were unsuccessful from a practical point of view. A UV-sensitive mutant, obtained by a semi-selective method using a rhizobiophage, although more sensitive to the mutagenic effects of UV than the wild-type, only yielded a slightly higher maximum induced frequency of rifampicin-resistant mutants because of its increased susceptibility to the lethal effects of UV. Attempts to transfer into this strain any one of a variety of plasmids known to enhance UV survival and mutagenesis were unsuccessful.

The concentrations of ammonium required to inhibit nodulation, 5mM, and nitrogen-fixation, 100mM, in the *R. trifolii*- *T. repens* symbiosis were determined prior to an attempt to obtain mutants of *R. trifolii* which were derepressed for nitrogen fixation in the presence of ammonium. Six mutants of *R. trifolii* resistant to the glutamate analogue methionine sulfoximine, an inhibitor of the enzyme glutamine synthetase (GS) which has been implicated in the regulation of nitrogen fixation, were not derepressed for nitrogen fixation. A mutant resistant to another glutamate analogue, methionine sulphone, was found to be completely ineffective. It
was thought that this strain might represent a class in which the regulatory but not catalytic activities of GS had been altered.

Six symbiotically defective mutants of *R. trifolii* P3 were isolated following transposon mutagenesis. Two of these were studied further and the transposon insertion of one was mapped on the chromosome of *R. leguminosarum* near to the allele *ser*-2.
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Chapter 1

GENERAL INTRODUCTION
All nitrogen in our foodstuffs is derived directly or indirectly from the atmospheric reservoir of nitrogen by nitrogen fixation. Non-biological nitrogen fixation contributes $85 \times 10^6$ tons per annum, the industrial fixation of nitrogen by the Haber-Bosch process contributing about $40 \times 10^6$ tons of nitrogen per annum and an estimated $45 \times 10^6$ tons being fixed by other abiological processes such as lightning, combustion and ozonisation (Burns and Hardy, 1975). Biologically-fixed nitrogen is thought to contribute about $175 \times 10^6$ tons per annum (Burns and Hardy, 1975), this being carried out by a variety of micro-organisms ranging from the obligatory symbiotic diazotrophs (as found in the Rhizobium-legume symbiosis), through various associative systems (e.g. Spirillum lipoferum in association with the roots of Digitaria decumbens, Döbereiner and Day, 1976) to the free-living nitrogen-fixing bacteria (e.g. Azotobacter) and blue-green algae (e.g. Nostoc).

In the period following the Second World War the availability of cheap fertiliser nitrogen produced industrially led to a decrease of interest in the Rhizobium-legume symbiosis and other nitrogen-fixing systems (see reviews by Quispel, 1974 and Evans, 1975). Now, a scarcity of appropriate sources of energy coupled with a variety of other factors such as population growth, changing dietary habits, inefficient fertiliser application etc. (see review by Hardy and Havelka, 1975) have led to a reconsideration of agricultural practices and a renewed interest in the possibility of increased dependence on biological nitrogen fixation (see review by Evans and Barber, 1977).

Study of the Rhizobium-legume symbiosis was begun in 1888 when Beijerinck succeeded in isolating root-nodule bacteria from leguminous plants and showed they were required for healthy plant development (see Stanier, Adelberg and Ingraham, 1977), although legumes were included in crop rotation as a method of supplying
nitrogen even in ancient agriculture, the evidence for this coming from excavated Neolithic and Bronze age sites in the near East and Europe (Zohary and Hopf, 1973). Subsequent studies have outlined the steps involved in symbiosis (see reviews by Nutman, 1955, 1969, and Dixon, 1969): *Rhizobium* spp. with specificities for particular groups of legumes (e.g. *Medicago sativa*, *Trifolium* spp., *Phaseolus* spp.), recognise their appropriate host and invade the root hairs of the plant (see review by Dart, 1974).

Plant lectins have recently been suggested to be involved in host-range specificity, and a model in which the lectins act as a bridge between similar antigenic determinants on the surface of the infecting rhizobium and the root hair surface of the host plant has been proposed (Dazzo and Hubbell, 1975). Nodules are formed by the multiplication of cortical cells which become packed with modified forms of the rhizobia known as bacteroids (see review by Libbenga and Bogers, 1974). It is this form of the bacterium which is responsible for the fixation of nitrogen in compatible symbioses (see review by Bergersen, 1974) although some *Rhizobium* spp. have recently been shown to fix nitrogen in pure culture (Keister, 1975; Pagan et al., 1975; Kurz and LaRue, 1975; McComb, Elliot and Dilworth, 1975). Nitrogen is fixed in all nitrogen-fixing bacteria, free-living or otherwise, by an elaborate enzyme system called nitrogenase which binds nitrogen gas and reduces it to ammonium (see review by Eady and Postgate, 1974).

An understanding of the genetic basis of biological nitrogen fixation may lead the way to increased protein production. In recent years, genetic studies on the bacterial contribution to the *Rhizobium*-legume symbiosis have been initiated (Beringer, Johnston and Wells, 1977; Johnston et al., 1978).

**Aims of this Study**

The aims of this study were firstly to establish the most effective mutagenic agent for *R. trifolii* and to find if the
susceptibility to mutation of this species could be increased in any way. A mutant strain of *Micrococcus radiodurans*, for example, was mutated by N-methyl-N′-nitro-N-nitrosoguanidine to a considerably greater extent than the wild-type (Tempest and Moseley, 1978) and an attempt was made to obtain a similar mutant of *R. trifolii*. Other approaches included the isolation of a UV (ultraviolet light)-sensitive mutant of *R. trifolii* P3 and experiments were carried out to try to transfer into this and other strains, plasmids known to enhance UV mutagenesis.

Secondly, a number of different methods were used in an attempt to isolate regulatory mutants of *R. trifolii*, the regulation of nitrogen-fixation in the symbiotic rhizobia not being as well understood as in the free-living nitrogen-fixing bacterium *Klebsiella pneumoniae*. Bearing these aims in mind, the rest of this introduction gives general background information on the work which has been carried out in these fields and the conclusions which have been drawn. More detailed information relating directly to the work which has been carried out in this study is given in the relevant chapters.

**Mutagenesis and mutants of *Rhizobium***

A genetic approach to the understanding of the *Rhizobium*-legume symbiosis and the future prospects for its genetic manipulation requires the production of mutant rhizobia. Types of mutant strains which may be isolated are those having altered symbiotic properties or those which are required for more general studies of the biochemical and genetic systems of the organism.

(a) **The indirect isolation of symbiotic mutants of *Rhizobium***

Mutants having altered symbiotic properties may be obtained by two possible approaches. The first is an indirect one whereby rhizobia isolated as having some altered function are further tested for alterations in their symbiotic properties.
Kleczkowska (1950, 1965), working with *R. trifolii* found that over 50% of phage-resistant mutants were ineffective (i.e. they formed nodules but did not fix nitrogen). Schwinghamer (1964, 1967) studied effectiveness as modified by resistance to antibiotics in *R. leguminosarum, R. meliloti* and *R. trifolii*. He observed that the acquisition of resistance to some antibiotics, e.g. neomycin or viomycin, was very closely correlated with loss of effectiveness, whereas resistance to some others, e.g. streptomycin, chloramphenicol, was very rarely associated with loss of effectiveness. In another study (Żelazna-Kowalska and Lorkiewicz, 1971), streptomycin resistance in *R. trifolii* was reported to have led to loss of infectivity. There was no adequate confirmation however that this "mutant" was a strain of *R. trifolii*. Schwinghamer (1968, 1969) also examined a number of mutants in four different species of *Rhizobium* which were resistant to metabolic inhibitors (D-amino acids or amino acid analogues). Almost half were defective in symbiosis being partially effective, ineffective or non-nodulating. Studies of this kind led to the suggestion that the bacterial cell wall and/or membrane are very important in the establishment of a successful symbiosis (Schwinghamer, 1969; Dénarié, Truchet and Bergeron, 1976).

Other indirectly-isolated symbiotic mutants are those having specific nutritional requirements (Schwinghamer, 1969; Lorkiewicz and Melke, 1970; Kowalski, 1971). Dénarié, Truchet and Bergeron (1976) found that loss of effectiveness was closely associated with mutation to requirements for purines, pyrimidines, leucine and isoleucine/valine whereas mutants with other auxotrophic requirements, e.g. arginine, glycine and cysteine, were rarely affected in their symbiotic properties. In some cases effectiveness was restored when the mutants were reverted to prototrophy (Dénarié, Truchet and Bergeron, 1976), in other cases revertants remained ineffective (Lorkiewicz et al., 1971).
It should be pointed out that in at least some of the studies mentioned so far, Rhizobium strains were not adequately characterised by the presence of genetic markers and therefore doubt can be cast on whether some "mutants" were directly derived from the claimed parental strains or indeed whether they were rhizobia at all.

Strains resistant to antibiotics which have not undergone alterations in their symbiotic properties can usefully be used in field and greenhouse studies, e.g. where the effects of inoculation of clover with rhizobia are being studied (Imshenetskii, Pariiskaya and Erraiz Lopez, 1970; Obaton, 1971; Schwinghamer and Dudman, 1973; Johnston and Beringer, 1975).

(b) The direct isolation of symbiotic mutants of Rhizobium

The second approach to the isolation of symbiotically defective mutants is a direct one involving screening of rhizobia in association with their host plant. Ineffective mutants have been isolated directly in a number of instances (Kleczkowska, 1985; Kowalski, 1970; Maier and Brill, 1976). Beringer, Johnston and Wells (1977) isolated three temperature-sensitive mutants of R. leguminosarum which were ineffective in plants grown at 26°C but effective in plants grown at 13°C. Three other mutants were ineffective at both temperatures. Non-infective mutants have also been found using a direct approach (Russell and Jones, 1973; Maier and Brill, 1976; Sanders, Carlson and Albersheim, 1978).

Other types of symbiotic mutants which have been obtained by the direct approach are those able to nodulate other hosts (Schwinghamer, 1962; O'Gara and Shanmugam, 1978) and those with increased effectiveness (Maier and Brill, 1977).

Some mutant strains have been examined in investigations of the regulation of nitrogen fixation (Tubb, 1976; Kondorosi et al., 1977b; Ludwig and Signer, 1977; O'Gara and Shanmugam, 1977). Most studies have been concerned with mutants having an altered
glutamine synthetase. This enzyme has been implicated in the control of nitrogen fixation, at least in the free-living organisms, as will be discussed later.

Mutant rhizobia strains are also used for more general purposes in genetic studies, e.g. those on transfer of R plasmids and formation of R-primes (Beringer, 1974; Johnston, Setchell and Beringer, 1978) and chromosome mapping (Meade and Signer, 1977; Kondorosi et al., 1977a; Beringer, Hoggan and Johnston, 1978; Casadesus and Olivares, 1979). They are also used in biochemical studies, e.g. the study of carbohydrate metabolism (Ronson and Primrose, 1979).

**Induction of mutations in Rhizobium**

Mutations in rhizobia and other bacteria can be obtained spontaneously or after treatment with a suitable mutagenic agent. Spontaneous mutants will usually only be obtained where they have a readily selectable phenotype such as resistance to an antibiotic or phage. Some of the mutant strains mentioned previously under the first category of symbiotic mutants were of this type. Induction of mutants can be used either to increase the number of mutants having a readily selectable phenotype or to increase the probability of finding mutants of a non-selectable phenotype in a screening procedure, e.g. that which is used to detect auxotrophs.

A variety of different mutagenic agents have been used to induce mutations in rhizobia. The three most commonly used are N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Schwinghamer, 1969; Beringer, 1974; Kondorosi et al., 1977a), ultraviolet light (UV) (Gupta and Kleczkowska, 1962; Kowalski, 1970; Kaushik and Venkataraman, 1972a) and ethyl methanesulphonate (EMS) (Schwinghamer, 1969; Kaushik and Venkataraman, 1972b; Meade and Signer, 1977). A number of other mutagens have also been employed in attempts to obtain mutant rhizobia. These include

(a) Problems associated with the determination of effectiveness of a mutagen

In only a few cases have specific mutagens been studied to determine their effectiveness in inducing mutations in rhizobia. This has usually been done by looking for induction of auxotrophs. Such an approach has a number of associated problems. Firstly, auxotrophs cannot be selected and therefore a screening procedure must be used to detect them. A large number of colonies are examined, of which a small percentage may be found to be auxotrophic. Consequently, the actual number of mutants found is low and the error inherent in any mutation frequency calculated is high. Secondly, probably as a result of the work involved in the detection of the mutants, the spontaneous level of auxotrophic mutants in the population is rarely given, and therefore no direct comparison of induced and spontaneous frequencies, to give a measure of the effectiveness of the mutagen, is possible. Other problems may arise due to errors in the replication process. For example, during replication of colonies onto minimal medium, some wild-type colonies may be determined as auxotrophic unless rechecking of results is carried out. The most satisfactory method for studying the effectiveness of mutagens is therefore to examine spontaneous and induced levels of readily detected strains mutant in only one function, e.g. antibiotic resistance.

(b) Studies on the effectiveness of a variety of mutagens

Bearing the aforementioned problems in mind, information indicative of the likely effectiveness of particular mutagens may nevertheless be obtained from such studies. Some of the earliest work of this kind was carried out by Jordan (1952). He looked at
the effect of X-rays, UV, uranium nitrate and diazomethane in the production of strains of *R. meliloti* with altered colony morphology and concluded that X-rays were the "most productive of variants". However, Schwinghamer (1960) could not detect auxotrophs following treatment of *Rhizobium* with X-rays and, in 1961, Schwinghamer and Dalmas, working with *R. trifolii*, showed that UV induced almost twice as many streptomycin-resistant mutants as X-rays at a comparable level of lethality representing respectively 7-fold and 4-fold increases over the spontaneous mutation frequency. The mutagenic effect of UV was also studied by Kaushik and Venkataraman (1972a) working with *R. trifolii*. They looked at the build-up of UV-resistance in populations as a result of cyclic UV irradiation and found that 14 out of 164 UV-resistant mutants were also streptomycin-resistant, an inexplicably high frequency. By way of contrast they found no auxotrophs in 3,780 colonies examined. In another study (Kaushik and Venkataraman, 1972b) they examined the ability of EMS to induce mutations to streptomycin-resistance and auxotrophy in *R. trifolii*. They isolated 13 auxotrophs from 813 survivors of the treatment, but found no streptomycin-resistant strains amongst these. Despite the fact that no spontaneous controls were included in the experiment, that the level even of induced streptomycin-resistant strains is likely to be considerably less than would be detected by the examination of 813 survivors, and that the frequency of streptomycin-resistant mutants could have been determined by plating on selective media, the experimenters concluded that "EMS was found to be effective in inducing auxotrophic mutants but ineffective for the induction of STM-resistant mutants"! Meade and Signer (1977) found that they could induce a 300-fold increase in the mutation frequency of rifampicin-resistant mutants in populations of *R. meliloti* following exposure to EMS.
UV was used by Lorkiewicz et al. (1971) to induce auxotrophic mutants of *R. trifolii*. They isolated 29 stable mutants but did not report the total number of colonies examined. In contrast, Beringer (1973) was unable to find auxotrophic mutants of *R. leguminosarum* among 500 survivors of UV treatment. Similarly, Imahenetskii, Parfiskaya and Erroz Lopez (1970) were unable to isolate auxotrophs of *R. meliloti* from among 712 survivors. These workers were also unable to isolate auxotrophs following treatment of *R. meliloti* with nitrosomethyl urea, ethylenimine and nitrous acid. The mutagenic effect of nitrous acid in *R. leguminosarum* was also studied by Beringer (1973). He found 2 auxotrophs amongst 422 survivors of the treatment and concluded that nitrous acid might be a useful mutagen in this species.

MNNG was studied by Raina and Modi (1969) and Beringer (1973). The former, working with *Rhizobium* cowpea, found a gradual increase in the number of auxotrophic mutants isolated with time of treatment to a final frequency of 20%. The latter found an approximately two-fold increase in the number of streptomycin-resistant mutants, and the number of auxotrophs detected rose from the spontaneous level of 0.08% to 0.46% in *R. leguminosarum* 300. In a later paper (Beringer, Johnston and Wells, 1977) he reports induction of 5% auxotrophs in *R. leguminosarum* 603.

the use of a transposon, Tn5, in the induction of mutants in *Rhizobium*

The use of a transposon to cause mutations in three *Rhizobium* species has recently been reported (Beringer et al., 1978). Transposons are discrete pieces of DNA which can insert into DNA replicons giving rise to non-leaky polar mutations (see review by Cohen, 1976). A transposon, Tn5, coding for kanamycin resistance was introduced into three *Rhizobium* species, *R. leguminosarum*, *R. trifolii* and *R. phaseoli*, from an
Escherichia coli donor by means of a plasmid pJB4J1. This plasmid is unstable in Rhizobium due to insertion within it of phage Mu, consequently any kanamycin-resistant ex-conjugants are likely to have arisen from a transposition event. Following "mutagenesis" with Tn5 the frequency of auxotrophy was about 0.3% and the range of auxotrophs found suggested that insertion was more-or-less random. Studies on the co-transduction of the auxotrophic mutants with kanamycin resistance provided strong evidence that the mutations were in fact due to transposon insertion rather than Mu insertion.

This method has the disadvantage that it cannot readily be used for producing mutations sequentially to give a multiply-marked strain. This would need the introduction of transposons carrying antibiotic resistances other than that to kanamycin, and would require the construction of suitable plasmid carriers, as for Tn5 on pJB4J1. The main advantage of this method is that it leads to the production of mutants which have a readily selectable phenotype, kanamycin-resistance, resulting from insertion of the transposon, in addition to the mutant phenotype. For example, symbiotic mutants of Rhizobium isolated by this method could readily be mapped, and perhaps transferred to other strains to elucidate the function which has been eliminated.

Applications for the genetic manipulation of mutants of Rhizobium

The ability to carry out genetic manipulations with strains of rhizobia having altered properties now leaves open the possibility of combining two or more desirable characteristics within one strain. These might be competitiveness, high nitrogen-fixing ability, ability to survive in soils of low pH, etc. Another desirable characteristic might be the ability to continue fixing nitrogen even when sufficient fixed nitrogen was available. Such strains would presumably fix more nitrogen in the long run than those
from which they were derived, and would also be less sensitive to effects of fertiliser which might, for example, be applied to aid development of a companion grass. The construction of such a strain requires an adequate knowledge of the regulation of nitrogen fixation.

The regulation of nitrogen fixation

(a) Introduction

In bacteria the presence of ammonium nitrogen in the culture medium normally represses the synthesis of a number of enzymes capable of supplying the cells with ammonium or glutamate from a variety of nitrogen-containing compounds such as histidine or proline (see review by Magasanik, 1977). Ammonium ions also inhibit nitrogen fixation in free-living micro-organisms such as *Klebsiella pneumoniae* (Tubb and Postgate, 1973) and *Azotobacter vinelandii* (Strandberg and Wilson, 1968). In *Rhizobium* ammonium inhibits nitrogen fixation both in its symbiotic state (Allos and Bartholomew, 1959; Pate and Dart, 1961; Kamberger, 1977) and as a free-living bacterium (Pagan et al., 1975; Evans and Keister, 1976). In the latter case a complete inhibition of fixation is not usually found (Pagan et al., 1975; Tubb, 1976) and the extent of inhibition observed may vary with cultural conditions such as oxygen tension (Bergersen et al., 1976).

Other sources of nitrogen such as amino acids, urea or nitrate are also capable of inhibiting nitrogen fixation. In most cases, however, this is thought to occur following their metabolism to yield ammonium. In the case of inhibition by nitrate ions, for instance, it has been shown that a mutant of *Azotobacter vinelandii* lacking nitrate reductase synthesises identical quantities of nitrogenase in the presence or absence of nitrate ions (Sorger, 1969).

It was thought, at one stage, that control of nitrogenase might be mediated by the induction of its synthesis by nitrogen
gas following depletion of a fixed nitrogen source. Ambiguous results were obtained at first because of the problem of removal of nitrogen gas from the test system (Strandberg and Wilson, 1968; Mahl and Wilson, 1968). Later experiments, in which levels of contaminating nitrogen gas were reduced to extremely low levels, suggested that it was very unlikely that this gas was acting as an inducer and that control of nitrogenase was simply in response to levels of ammonium in the environment (Parejko and Wilson, 1970; Daesch and Mortenson, 1972).

Evidence from a number of experiments suggests that high concentrations of ammonium repress the synthesis of nitrogenase but do not affect its activity (Mahl and Wilson, 1968; Daesch and Mortenson, 1972; Davis et al., 1972) and that this occurs at the level of messenger RNA transcription (Tubb and Postgate, 1973).

In the symbiotic rhizobia, the situation may be somewhat more complicated, as some recent evidence has suggested that the effects of ammonium on nitrogen fixation may be mediated indirectly. Houwaard (1978) found a 20-40% decrease in acetylene reduction by nodulated pea plants following addition of 20mM ammonium chloride, but found no drop in the nitrogenase activity of bacteroids isolated from these plants. He suggested that inhibition of fixation was due to a reduced supply of photosynthates to the bacteroids. Bisseling et al. (1978) found a similar situation but correlated the decreased nitrogenase activity in intact nodules with a decrease in the amount of leghaemoglobin within the nodules.

(b) The regulation of nitrogen fixation in the free-living nitrogen-fixing bacterium Klebsiella pneumoniae

In bacteria, including free-living nitrogen-fixing bacteria, the assimilation of $\text{NH}_4^+$ can occur by either of two pathways (Meers, Tempest and Brown, 1970; Nagatani, Shimizu and Valentine, 1971):
The first pathway operates only at high extracellular ammonium concentrations (>1mM) by the action of the enzyme glutamate dehydrogenase (GDH; EC 1.4.1.2.). At low concentrations of ammonium, assimilation requires the action of two enzymes, glutamine synthetase (GS; EC 6.3.1.2.) followed by glutamate synthase (GOGAT; EC 2.6.1.53.). This second pathway is important in the assimilation of ammonium produced by the fixation of gaseous nitrogen (Nagatani, Shimizu and Valentine, 1971).

A number of enzymes involved in bacterial nitrogen assimilation, including nitrogenase, are regulated by GS (see reviews by Magasanik et al., 1974; Magasanik, 1977) which responds to the levels of available ammonium. The nature of this regulation has been elucidated by work on E. coli and Klebsiella aerogenes and can be summarized as follows (Figure 1-1; for details see Magasanik, 1977).

A high concentration of available ammonium, >1mM, added to cells growing in a medium with a low concentration of ammonium, <1mM, leads to a rapid inactivation of the enzyme. This occurs by the addition of adenylyl (AMP) groups to the enzyme following activation of a complex system which carries this out. The activation of this system depends on the ratio of α-ketoglutarate to glutamine within the cell; this ratio will, of course, depend on the amount of ammonium being assimilated. The available evidence strongly suggests that the inactive GS acts as a repressor of its own synthesis and thus the level of enzyme within the cell falls. It has been shown that unadenylylated GS activates the transcription of the histidine utilisation (hut) genes of Salmonella...
Low NH₄⁺ (<1mM)
High ratio, α-ketoglutarate:glutamine

GS
Catalytically active
Activates transcription

High NH₄⁺ (>1mM)
Low ratio, α-ketoglutarate:glutamine

GS-AMP
Catalytically inactive
Represses transcription

Structural genes for nitrogen assimilation enzymes

GlnA, structural gene for GS

Other regulatory controls
1. Amino acids
Asparagine + glutamine
2. Nif-specific repressor
binds at high NH₄⁺ preventing transcription
3. Other regulatory controls?

Structural genes for nitrogenase in the nif operon

Figure 1-1. A model for the regulation of nitrogen fixation in *Klebsiella pneumoniae*
typhimurium in vitro (Tyler, Deleo and Magasanik, 1974) and presumably does likewise for the genes of the other enzymes which it regulates.

Evidence for the involvement of GS in the regulation of nitrogenase in free-living nitrogen-fixing bacteria (see Figure 1-1 for summary) has come from studies with bacteria having an altered or no GS (see reviews by Shanmugam and Valentine, 1975; Brill, 1975). Streicher et al. (1974) found that a glutamate-requiring auxotroph of K. pneumoniac, which could not synthesise catalytically active GS, was also unable to synthesise nitrogenase during nitrogen-limited growth. Complementation with an E. coli episome, F′133, coding for active GS restored ability to synthesise nitrogenase. In addition, they found that, following transduction of a mutation causing constitutive synthesis of GS (GlnC− phenotype) from K. aerogenes to K. pneumoniac, nitrogenase was still synthesised in the presence of a high ammonium concentration, albeit at reduced levels. Support for these observations came from Tubb (1974) who transferred an F′nif factor into GlnC− K. aerogenes strains and found constitutive synthesis of nitrogenase in the presence of ammonium.

These studies have led to the construction of derepressed strains of K. pneumoniac (Shanmugam and Valentine, 1974; Shanmugam, Chan and Morandi, 1975; see also review by Shanmugam, Morandi and Valentine, 1977) some of which excrete fixed nitrogen as ammonium due to blocks in the pathway for its assimilation. Further work on some of these strains showed that their nitrogenase could still be repressed by certain combinations of amino acids such as L-glutamine and L-asparagine or L-glutamine and ammonium (Shanmugam and Morandi, 1976; Shanmugam et al., 1977). It was suggested on this evidence that the overall mechanism of nitrogenase regulation requires the conversion of ammonium to the level of amino acids.
Recent evidence has now indicated that GS may not be the sole regulator of nitrogenase synthesis (Ausubel, Margolskee and Maizels, 1977; Ausubel et al., 1977). Mutants of *K. pneumoniae* have been isolated in which synthesis of nitrogenase is independent of the level of GS within the cell but is still inhibited by ammonium. The evidence suggested that these probably carry promoter mutations, e.g. they were cis-dominant and linked to the *nif* genes, in which *nif* transcription is rendered independent of GS activation. The finding that ammonium was still effective in repression, however, led to the postulation of a second controlling element, a *nif*-specific repressor which binds in the presence of ammonium and blocks transcription. Thus, regulation of the *nif* genes would be mediated by positive control by GS, activation of transcription occurring at low concentrations of ammonium, and negative control by an ammonium-sensitive *nif*-specific repressor which blocks transcription at high concentrations of ammonium (see Figure 1-1). This model was further supported by the fact that some *nif* mutants, whose mutation mapped in the GS structural gene but nevertheless had biosynthetically active GS, could in fact be derepressed at very low levels of ammonium. According to the model these mutants have a GS which has retained its biosynthetic but lost its regulatory properties. Control of the *nif* genes is mediated solely by the ammonium-sensitive *nif*-specific repressor and therefore the block to transcription is lifted in the absence of the positive activator only at very low concentrations of ammonium. It has been suggested, although there is no supporting evidence, that the *nif L* gene may code for the *nif*-specific repressor (MacNeil et al., 1978). Work on the mapping of the *nif* genes in *K. pneumoniae* suggests that the regulation of nitrogen fixation may be very complex. Roberts et al. (1978) have tentatively assigned regulatory gene products to six of the genes found in the *nif* gene cluster.
(c) The regulation of nitrogen-fixation in Rhizobium

In Rhizobium, the regulation of ammonium assimilation via either of two pathways involving different ammonium-assimilating enzymes has not been firmly established. Brown and Dilworth (1975) reported that ammonium assimilation in three species of free-living rhizobia (R. leguminosarum, R. trifolii and R. japonicum) occurs by either of the two accepted pathways (GS/GOGAT- or GDH-catalysed) depending on the ammonium concentrations, as found for Klebsiella. Kondorosi et al. (1977b) working with R. meliloti, obtained evidence only for the first of these pathways which operated at both high and low concentrations of ammonium, although the levels of GS were reduced at the higher concentrations. They were also unable to establish the presence of an adenylylation-deadenylylation system for GS. Tronick, Ciardi and Stadtman (1973), however, provided evidence for such a system in R. japonicum.

Bergersen and Turner (1967) found that most nitrogen fixed by bacteroids is excreted by them as ammonium. This observation is supported by a body of evidence which suggests that the levels of the ammonium assimilation enzymes are reasonably high within the supernatant (plant) fraction prepared from root nodules but not within the bacteroids (Brown and Dilworth, 1975; Robertson, Warburton and Farnden, 1975; Kurz, Rokosh and LaRue, 1975). Dunn and Klucas (1973) claimed to have detected adequate levels of ammonium assimilation enzymes within the bacteroids of R. japonicum although levels were still higher in the nodule supernatant fraction. Their anomalous result might be explained by the use of an assay which could also measure inactive enzyme.

The role of ammonium and GS in the regulation of nitrogen fixation in rhizobia has not been clarified. Figure 1-2 shows some possible regulatory controls of nitrogen fixation in the symbiotic Rhizobium and these are discussed in the remainder of
Plant controls of nitrogenase synthesis e.g. GS?

1. Low $\text{NH}_4^+$  
   GS $\rightarrow$ GS-AMP  
   Catalytically active  
   Activates transcription  

2. Other regulatory controls  
   e.g. nif-specific repressor?

$nif$ operon? including genes for nitrogenase

$\text{NH}_4^+ \rightarrow$ glutamate

Plant controls of nitrogenase activity  
   e.g. supply of photosynthetic  
   amount of nodule leghaemoglobin

$\text{NH}_4^+ \rightarrow$ glutamine $\rightarrow$ glutamate $\rightarrow$ amino acids

Figure 1-2. Possible regulatory controls of nitrogen fixation in symbiotic Rhizobium (based on Shanmugam et al., 1978)
this section. In the free-living nitrogen-fixing rhizobia, although ammonium at concentrations sufficient to cause complete inhibition of fixation in *Klebsiella* may reduce nitrogenase activity to a certain extent depending on the culture conditions, this is not a complete inhibition and in a sense the nitrogenase enzyme can be said to be derepressed (Tubb, 1976). Tubb also observed that L-glutamate inhibited utilisation of exogenous ammonium, and also ammonium produced by nitrogen fixation. Similar results were obtained by O'Gara and Shanmugam (1976) which led to their proposing a model for symbiotic nitrogen fixation (see also Shanmugam et al., 1978) by analogy with derepressed ammonium-excreting strains of *K. pneumoniae*. They suggested that during symbiotic nitrogen fixation the nitrogen-fixing genes are derepressed and the ammonium assimilatory genes of the bacteria are repressed by amino acids (notably glutamate) supplied by the plant. This results in excretion of ammonium from the bacteroids into the nodule cytosol where assimilation to the level of amino acids occurs, these then being translocated to the rest of the plant.

This model and the evidence supporting it suggests it is unlikely that regulation of the nitrogenase enzyme in symbiotic nitrogen fixation is mediated by bacterial GS. This is also suggested by experiments carried out by Scowcroft, Gibson and Pagan (1976) who found that, when nitrogen fixation in free-living *Rhizobium* cowpea was inhibited by ammonium, levels of GS and GOGAT were unaffected. Bishop et al. (1976) showed that inhibiting concentrations of ammonium had no effect on adenylylation of GS from isolated bacteroids of *R. japonicum* whereas an effect was observed in the free-living nitrogen-fixing bacteria.

Genetic evidence, however, has been obtained suggesting that GS may play a regulatory role in nitrogen fixation. Kondorosi et al. (1977b) isolated a glutamate-requiring mutant and a glutamine-requiring mutant of *R. meliloti*. The former lacked
GOGAT but was still effective in symbiosis (this supporting the previously-mentioned model in which bacterial ammonium assimilatory enzymes are not involved in symbiosis). The latter had a low level of GS and was completely ineffective suggesting that the enzyme is either required for a late stage of symbiosis or for the activation of nitrogenase. A glutamine-requiring mutant of Rhizobium cowpea lacked nitrogenase activity in both the free-living and symbiotic form. Two out of six revertants to prototrophy regained nitrogenase activity, the other four representing a class which may have gained catalytic but not regulatory activity (Ludwig and Signer, 1977).

The control of nitrogen fixation in the symbiotic rhizobia may turn out to have some similarities to that in the free-living bacteria (see Figure 1-2). There remains the possibility, however, that there are other regulatory controls arising from the symbiotic association which may be partly or wholly plant-mediated. As mentioned previously, for instance, supply of photosynthates or amounts of leghaemoglobin in the nodule may affect nitrogen fixation. It is also possible that the plant may play a direct role in the regulation of synthesis and activity of nitrogenase itself. Further research may elucidate some of the mechanisms involved.
Chapter 2

GENERAL MATERIALS AND METHODS
Bacteria

The species and strains of bacteria used in this project, along with their relevant phenotype or genotype and source are listed in Tables 2-1 to 2-3. Plasmids are listed in Table 2-4. Some of these were transferred to hosts other than the one in which they were received as can be seen from these tables.

Viruses

A phage, designated Rt1, infective for R. trifolii P3 was isolated during the course of this work.

Plants

All experiments were carried out with Trifolium repens cv. Huia.

Media

Media for growth of Rhizobium

(a) TY (Beringer, 1974) $g^{-1}$

- Difco Bacto-tryptone 5
- Difco yeast extract 3
- CaCl$_2$· 6H$_2$O 1.3
- Distilled water to 1 l

(b) SY (Beringer, 1974) $g^{-1}$

- K$_2$HPO$_4$ 0.22
- Distilled water to 1 l

The following were sterilised separately and added to the medium immediately prior to use.
<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Relevant phenotype or genotype (a) (b)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCR1</td>
<td>wild type</td>
<td>Rothamsted Experimental Station (c)</td>
</tr>
<tr>
<td>RCR4</td>
<td>wild type</td>
<td>&quot;</td>
</tr>
<tr>
<td>RCR5</td>
<td>wild type</td>
<td>&quot;</td>
</tr>
<tr>
<td>RCR32</td>
<td>wild type</td>
<td>&quot;</td>
</tr>
<tr>
<td>RCR49</td>
<td>wild type</td>
<td>&quot;</td>
</tr>
<tr>
<td>FA6</td>
<td>wild type</td>
<td>Prof. A.J. Holding (d)</td>
</tr>
<tr>
<td>DC01t</td>
<td>rif-1 (derived from FA6)</td>
<td>This thesis</td>
</tr>
<tr>
<td>1DL</td>
<td>wild type</td>
<td>Prof. A.J. Holding</td>
</tr>
<tr>
<td>DC001t</td>
<td>rif-1 (derived from 1DL)</td>
<td>This thesis</td>
</tr>
<tr>
<td>P3</td>
<td>wild type</td>
<td>Prof. A.J. Holding</td>
</tr>
</tbody>
</table>

**Derivatives of P3**

| DC1t               | rif-1                                 | This thesis |
| DC2t               | str-1                                 | "          |
| DC3t               | rif-1 (R68.45)                        | "          |
| DC4t               | rif-1 str-2 (R68.45)                  | "          |
| DC5t               | uvs-1                                 | "          |
| DC6t               | uvs-1 rif-2                           | "          |
| DC7t               | uvs-1 spc-1                           | "          |
| DC8t               | uvs-1 rif-2 (R68.45)                  | "          |
| DC9t               | cys-1                                 | "          |
| DC10t              | met-1                                 | "          |
| DC11t              | met-2                                 | "          |
| DC12t              | trp-1                                 | "          |
| DC13t              | ade-1                                 | "          |
| DC14t              | rif-1 cys-2::Tn5                       | "          |
| DC15t              | rif-1 met-3::Tn5                       | "          |
| DC16t              | rif-1 trp-2::Tn5                       | "          |

Contd./
### Table 2-1 (Contd.)

<table>
<thead>
<tr>
<th>Strain designation</th>
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<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC17t</td>
<td>rif-1 ade-2::Tn5</td>
<td>This thesis</td>
</tr>
<tr>
<td>DC18t</td>
<td>rif-1 leu-1::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC19t</td>
<td>rif-1 his-1::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC20t</td>
<td>rif-1 Eff-1::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC21t</td>
<td>rif-1 Eff-2::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC22t</td>
<td>rif-1 Eff-2::Tn5 (pJB3JI)</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC23t</td>
<td>rif-1 Eff-3::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC24t</td>
<td>rif-1 Eff-4::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC25t</td>
<td>rif-1 Eff-5::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC26t</td>
<td>rif-1 Eff-6::Tn5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC27t</td>
<td>rif-1 MSX-1</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC28t</td>
<td>rif-1 MSX-2</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC29t</td>
<td>rif-1 MSX-3</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC30t</td>
<td>rif-1 MSX-4</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC31t</td>
<td>rif-1 MSX-5</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC32t</td>
<td>rif-1 MSX-6</td>
<td>&quot;</td>
</tr>
<tr>
<td>DC33t</td>
<td>rif-1 MS-1</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

(a) Abbreviations: Antibiotic resistance - rif-rifampicin; str-streptomycin; spc-spectinomycin. Growth requirements - cys-cysteine; met-methionine; trp-tryptophan; ade-adenine; leu-leucine; his-histidine. Others - uvs-UV-sensitivity; Eff - symbiotically defective; MSX - resistant to methionine sulfoximine; MS - resistant to methionine sulphone.

(b) Plasmids carried by these strains are in parenthesis.

(c) Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire.

(d) Department of Agricultural and Food Microbiology, Queen's University of Belfast, Belfast, Northern Ireland.
Table 2-2. Strains of *Rhizobium leguminosarum* used

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Relevant genotype (a)</th>
<th>Map section (b)</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>1860</td>
<td>met-12 rib-2 str-69</td>
<td>1</td>
<td>Dr. J.E. Beringer (c)</td>
</tr>
<tr>
<td>1433</td>
<td>ade-27 rib-2 str-69</td>
<td>2</td>
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<tr>
<td>1628</td>
<td>ura-14 ade-27 str-75</td>
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<td>1062</td>
<td>ura-14 trp-16 str-86</td>
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</tr>
<tr>
<td>1056</td>
<td>ura-14 met-14 str-84</td>
<td>5</td>
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</tr>
<tr>
<td>1783</td>
<td>ser-2 met/cys-19</td>
<td>6</td>
<td>&quot;</td>
</tr>
<tr>
<td>1784</td>
<td>ser-2 ade-88</td>
<td>7</td>
<td>&quot;</td>
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<tr>
<td>1629</td>
<td>cys-8 ura-14 ade-88 str-69</td>
<td>8</td>
<td>&quot;</td>
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<tr>
<td>1840</td>
<td>cys-8 ura-14 str-69</td>
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</tr>
<tr>
<td>DC11</td>
<td>ser-2 met/cys-19 str-1</td>
<td>6</td>
<td>This thesis, streptomycin-resistant derivative of 1783</td>
</tr>
<tr>
<td>DC21</td>
<td>ser-2 ade-88 str-2</td>
<td>7</td>
<td>This thesis, streptomycin-resistant derivative of 1784</td>
</tr>
<tr>
<td>300</td>
<td>wild type</td>
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<td>This thesis, spectinomycin derivative of 300</td>
</tr>
<tr>
<td>DC31</td>
<td>spc-1</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

(a) Abbreviations: Growth requirements - rib - riboflavin, ura - uracil, ser - serine.
(b) See Figure 2-2.
(c) John Innes Institute, Norwich, East Anglia.
Table 2-3. Other species used

<table>
<thead>
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<th>Strain designation</th>
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<th>Source or reference</th>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>W677</td>
<td>thr leu thi lac (R751-SU2)</td>
<td>Dr G A Jacoby (b)</td>
</tr>
<tr>
<td>1830</td>
<td>pro met nal (pJB4J1)</td>
<td>Dr J E Beringer</td>
</tr>
<tr>
<td>1843</td>
<td>pro met nal (pJB3J1)</td>
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</tr>
<tr>
<td>551</td>
<td>pro met nal (R702)</td>
<td>&quot;</td>
</tr>
<tr>
<td>J5-3 (R68,45)</td>
<td>pro met nal (R68,45)</td>
<td>&quot;</td>
</tr>
<tr>
<td>J5-3 (RP4)</td>
<td>pro met nal (RP4)</td>
<td>Dr B E B Moseley (c)</td>
</tr>
<tr>
<td>J5-3 (R702)</td>
<td>pro met nal (R702, pKM101)</td>
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</tr>
<tr>
<td>(pKM101)</td>
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</tr>
<tr>
<td>24</td>
<td>thr leu str</td>
<td>Dr B E B Moseley</td>
</tr>
<tr>
<td>24-1</td>
<td>thr leu str rif-1</td>
<td>This thesis</td>
</tr>
<tr>
<td>24-2</td>
<td>thr leu str rif-1(pKM101)</td>
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</tr>
<tr>
<td>24-3</td>
<td>thr leu str (pKM101)</td>
<td>&quot;</td>
</tr>
<tr>
<td>CSH-29</td>
<td>trp thi</td>
<td>Cold Spring Harbor Kit (d)</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
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<td></td>
</tr>
<tr>
<td>PU21(pMG1)</td>
<td>FP- ilv B112 leu-1 str-1 rif (pMG1)</td>
<td>Dr G A Jacoby</td>
</tr>
<tr>
<td>PU21(R931)</td>
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<tr>
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<tr>
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<tr>
<td><strong>Pseudomonas putida</strong></td>
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<td></td>
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<tr>
<td>AC34(R751-SU2)</td>
<td>ade(R751-SU2)</td>
<td>Dr G A Jacoby</td>
</tr>
<tr>
<td><strong>Salmonella typhimurium</strong></td>
<td></td>
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<tr>
<td>TA100</td>
<td>his bi Δ uvrB(pKM101)</td>
<td>McCann et al. (1975)</td>
</tr>
<tr>
<td><strong>Rhizobium melloti</strong></td>
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</tr>
<tr>
<td>2011</td>
<td>wild type</td>
<td>Rothamsted Experimental Station</td>
</tr>
<tr>
<td><strong>Rhizobium phaseoli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8086</td>
<td>wild type</td>
<td>Dr J E Beringer</td>
</tr>
</tbody>
</table>

(a) Abbreviations: Antibiotic resistance - nal-nalidixic acid, Growth requirements - pro-proline, thi-thiamin, ilv-isoleucine or valine, bi-biotin. Others - uvr-UV sensitive.
(b) Massachusetts General Hospital, Boston, Massachusetts, U.S.A.
(c) Department of Microbiology, University of Edinburgh.
(d) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, U.S.A.
Table 2-4. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Compatibility group</th>
<th>Phenotype (a)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>R68.45</td>
<td>P1</td>
<td>Ap Km Tc</td>
<td>Dr J E Beringer</td>
</tr>
<tr>
<td>pJB4JI</td>
<td>P1</td>
<td>Gm Sp : Sm::Mu::Tn5 (b)</td>
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</tr>
<tr>
<td>pJB3JI</td>
<td>P1</td>
<td>Ap Tc</td>
<td>&quot;</td>
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<tr>
<td>R702</td>
<td>P1</td>
<td>Km Sm Su Tc</td>
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<td>RP4</td>
<td>P1</td>
<td>Ap Km Tc</td>
<td>Dr B E B Moseley</td>
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<td>pKM101</td>
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<td>Ap UV</td>
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<td>pMG1</td>
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<td>Gm Sm Su Hg UV</td>
<td>Dr G A Jacoby</td>
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<td>R931</td>
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<td>R2</td>
<td>P9</td>
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<td>R751-SU2</td>
<td>-</td>
<td>Tm Ap Km Sm Su UV</td>
<td>&quot;</td>
</tr>
</tbody>
</table>


(b) Streptomycin resistance is low level.
Addition | Stock solution g ml\(^{-1}\) | ml added l\(^{-1}\)
--- | --- | ---
\(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}\) | 0.1 | 1
\(\text{CaCl}_2\) | 0.04 | 1
\(\text{FeCl}_3 \cdot 6\text{H}_2\text{O}\) | 0.033 | 1
\(\text{Na succinate}\) | 0.27 | 5
\(\text{Na glutamate}\) | 0.22 | 5
\text{Biotin}\) | | |
\text{Thiamine hydrochloride}\) | 0.001 | 1
\text{Calcium pantothenate}\) | | |

(c) YEM (Vincent, 1970)

\(\text{K}_2\text{HPO}_4\) | 0.5 | |
\(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}\) | 0.2 | |
\(\text{NaCl}\) | 0.1 | |
\text{Mannitol}\) | 10.0 | |
\text{Difco yeast extract}\) | 0.4 | |
Distilled water to 1 l

Media for growth of other species

(a) Nutrient broth and agar

\(\text{Oxoid nutrient broth}\) | 25 | |
\text{(Oxoid Ltd., Basingstoke, Hants.)}\) | | |
Distilled water to 1 l

(b) M9 (Clowes and Hayes, 1968)

M9 salts (x 10 concentrate)

\(\text{Na}_2\text{HPO}_4\) | 60 | |
\(\text{KH}_2\text{PO}_4\) | 30 | |
\(\text{NaCl}\) | 5 | |
\(\text{NH}_4\text{Cl}\) | 10 | |
Dissolved in order as above.

- M9 salts x 10 100ml
- 20% glucose 20ml
- 0.1M MgSO$_4$ 10ml
- 0.01M CaCl$_2$ 10ml

Distilled water added to 1 l

Each component autoclaved separately and mixed aseptically just before use.

(c) Seedling agar and nutrient solution (Jenson, 1942)

\[
\begin{align*}
\text{CaHPO}_4 & \quad 1.0 \\
\text{K}_2\text{HPO}_4 & \quad 0.2 \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.2 \\
\text{NaCl} & \quad 0.2 \\
\text{FeCl}_3 & \quad 0.1 \\
\end{align*}
\]

Trace elements were then added as 1ml $^{-1}$ of a stock solution containing

- Bo 0.05%
- Mn 0.05%
- Zn 0.005%
- Mo 0.005%
- Cu 0.002%

The agar medium was dispensed into 150 x 19mm test tubes in 8ml amounts, sterilised, and the tubes allowed to sit at an angle while the agar set, thus forming a slope.

Liquid medium was made up at half the strength of the solidified medium.

Sterilisation of media, filters etc. was carried out in an autoclave at 121°C (1.05 kg cm$^{-2}$ steam pressure) for 15 minutes. Solutions of vitamins, amino acids, bases and antibiotics were
sterilised by passing them through a Millipore HAWP membrane filter, 0.45 μm pore size (Millipore UK Ltd., Wembley, Middlesex).

Media were solidified as required with 15 g l⁻¹ Difco Bacto-agar (Difco Laboratories, Detroit, Michigan). Agar media were made up in 1 l amounts in Roux flasks. Following sterilisation they were either stored in a 55 °C incubator until used (within three days) or allowed to set and melted when required by autoclaving as above. Melted media were poured into plastic petri dishes (Sterilin, Teddington, Middlesex). All plates were dried before use.

When supplements were required they were added to the agar media immediately prior to pouring, to give the concentrations shown in Tables 2-5 and 2-6, unless otherwise indicated. Liquid media were made up in 100ml or 200ml amounts in medical flats.

Buffers
(a) Phosphate buffer, 0.067M, pH 7.0

\[ \text{g l}^{-1} \]

\[ \text{KH}_2\text{PO}_4 \quad 4.56 \]

\[ \text{Na}_2\text{HPO}_4 \quad 4.75 \]

(b) Phosphate/EDTA, pH 7.5

\[ \text{g l}^{-1} \]

\[ \text{K}_2\text{HPO}_4 \quad 2.82 \]

\[ \text{KH}_2\text{PO}_4 \quad 0.52 \]

\[ \text{Na}_2\text{EDTA} \quad 3.36 \]

(c) Acetate buffer, 0.1M, pH 4.5

\[ \text{g l}^{-1} \]

\[ \text{CH}_3\text{COONa} \quad 4.02 \]

\[ \text{CH}_3\text{COOH} \quad 2.95 \]
Table 2-5. Supplements for minimal medium (based on Clowes and Hayes, 1968)

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<thead>
<tr>
<th>Supplement</th>
<th>Final concentration (µg ml(^{-1}))</th>
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<td>Amino acids</td>
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<td>Bases</td>
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</tr>
<tr>
<td>Sodium thiosulphate</td>
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</tr>
<tr>
<td>Choline</td>
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<tr>
<td>Inositol</td>
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<tr>
<td>Riboflavin</td>
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</tr>
<tr>
<td>Folic acid</td>
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</tr>
<tr>
<td>Nicotinic acid</td>
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</tr>
<tr>
<td>Pyridoxin</td>
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</tr>
</tbody>
</table>

Table 2-6. Antibiotic supplements for media

<table>
<thead>
<tr>
<th>Antibiotic (a)</th>
<th>Stock solution (mg ml(^{-1})) (b)</th>
<th>Solvent</th>
<th>Concentration in media (µg ml(^{-1}))</th>
<th>M.I.C. (µg ml(^{-1})) (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>25</td>
<td>Dimethyl sulphoxide</td>
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<td>0.5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100</td>
<td>Distilled water</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>Distilled water</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5</td>
<td>Distilled water</td>
<td>2.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>Dilute NaOH</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100</td>
<td>Distilled water</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>50</td>
<td>Distilled water</td>
<td>50</td>
<td>ND (d)</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>100</td>
<td>Distilled water</td>
<td>100</td>
<td>ND</td>
</tr>
</tbody>
</table>

(a) Rifampicin, kanamycin, ampicillin and tetracycline were obtained from Sigma Chemical Co. Ltd., London; streptomycin from Glaxo Laboratories Ltd., England; nalidixic acid from Calbiochem, San Diego, U.S.A.; carbenicillin from Beecham Research Laboratories, Brentford, England; and spectinomycin from Upjohn Ltd., Crawley, Sussex.
(b) All stock solutions except tetracycline, ampicillin and carbenicillin were stored in the dark at 4°C. These others were made up immediately prior to use.

(c) Minimum inhibitory concentration for R. trifolii P3. This was determined as the lowest concentration of antibiotic causing complete inhibition of growth on an agar plate.

(d) ND - not determined.
(d) Sodium saline citrate (SSC), pH 7.0

\[
\begin{align*}
\text{NaCl} & \quad 9.0 \\
\text{Na}_3 \text{citrate} & \quad 4.0 \\
\end{align*}
\]

(e) Phage buffer, pH 7.0 (Clowes and Hayes, 1968)

\[
\begin{align*}
\text{Na}_2 \text{HPO}_4 & \quad 7.0 \\
\text{KH}_2 \text{PO}_4 & \quad 3.0 \\
\text{NaCl} & \quad 5.0 \\
0.1\text{M MgSO}_4 & \quad 10\text{ml} \\
0.01\text{M CaCl}_2 & \quad 10\text{ml} \\
\end{align*}
\]

(f) TES buffer, pH 8.0

\[
\begin{align*}
\text{Tris} & \quad 6.05 \\
\text{EDTA} & \quad 1.86 \\
\text{NaCl} & \quad 2.92 \\
\end{align*}
\]

**Chemicals**

Ethyl methanesulphonate (EMS) and methyl methanesulphonate (MMS), obtained from Eastman Kodak and Co. (Rochester, New York), were stored in the dark at room temperature. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in phosphate buffer pH 7.0 at a concentration of 1mg ml\(^{-1}\) and stored at -20°C. Mitomycin C (MTC; Sigma Chemical Co. Ltd., London) and decarbamoyl mitomycin C (DMTC; a gift from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) were stored in phosphate buffer at a concentration of 100\(\mu\)g ml\(^{-1}\) at 4°C. L-methionine-DL-sulphoximine and DL-methionine sulphone (Sigma Chemical Co. Ltd.) were respectively stored dessicated at 4°C, and in the dark at room temperature.
The radioisotope $^3\text{H}^\text{H}$ thymidine (Radiochemical Centre, Amersham) was stored in the dark at 4°C. Lysozyme (E.C. 3.2.1.17, Grade 1; Sigma Chemical Co. Ltd.) was stored in a dessicator at 4°C and dissolved in phosphate buffer, at the concentrations indicated elsewhere, immediately prior to use.

**Handling of cultures**

Stock cultures were maintained as suspensions containing $10^9$ viable units (v.u.) ml$^{-1}$ in 20% glycerol in TY broth at -20°C. Liquid suspensions of crosses and mutagenised strains were also stored in this way. Cultures used frequently were also maintained on agar plates at 4°C and were sub-cultured at six week intervals by streaking from a single representative colony. Plasmid-containing strains used routinely were plated on medium supplemented with at least one of the antibiotics for which the plasmid encoded resistance.

Liquid cultures were grown in 10-20ml amounts in 250ml Erlenmeyer flasks on an orbital incubator or a reciprocal shaking water bath. *Rhizobium* strains and *Pseudomonas putida* were incubated at 30°C. *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* were incubated at 37°C.

Growth was measured by following changes in the turbidities of cultures grown in flasks with fitted sidearms. Turbidity was measured in a nephelometer (Evans Electroselenium Ltd., Halstead, Essex) using an orange filter and could be correlated with viable numbers of bacteria (see Figure 2-1).

Dilutions of cultures for counts of viable numbers were carried out in either TY broth or phosphate buffer. These were 10-fold (0.5ml into 4.5ml) or 100-fold (0.1ml into 9.9ml).
Figure 2-1. The relationship between viable numbers and turbidity
Isolation of genetically marked strains

Antibiotic resistant strains were isolated by plating a suspension of the parental strain containing about $10^{10}$ v.u. ml$^{-1}$ (obtained by centrifuging a late log-phase culture and resuspending in one tenth the original volume) onto plates containing the appropriate antibiotic. Single colonies arising after 4 days incubation were restreaked for purification on the selective medium.

Auxotrophic strains were isolated following mutagenesis of the parental strain (see Chapter 3) in the following manner.

The mutagenised culture was plated on TY medium to give a series of plates (usually about 50) with about 50 colonies per plate. These were replicated onto SY minimal medium using sterile velvet. Putative auxotrophs were identified by their inability to grow on this medium and their nutritional requirements identified by streaking onto a series of plates each supplemented with one of the compounds shown in Table 2-5. Strains which did not grow on any of the supplemented plates were presumed to have more than one nutritional requirement.

Preparation of high titre phage lysates

High titre phage lysates were obtained by either of two methods. In the first method a suspension of the phage was plated by the agar layer method (see below) to yield confluent plaques. This layer was then scraped off into 3ml broth, thoroughly shaken and 0.2ml chloroform added to kill any remaining bacteria. The suspension was shaken again, centrifuged at 3,000 rpm on a bench centrifuge (MSE Angle Centrifuge) for 2-3 minutes. The supernatant was removed and stored at 4°C. This method usually gave a phage lysate containing about $10^9$ plaque-forming units (p.f.u.) ml$^{-1}$. In the second method a suspension of phage was added to a log-phase culture of the bacteria to give a multiplicity of infection (m.o.i.) of about 3. After approximately 3 hours, lysis could be observed by a drop in the
turbidity of the culture. One drop of chloroform per ml of culture was added and the mixture shaken before centrifuging and treating as before. This method usually yielded a lysate of about $10^{10}$ p.f.u. ml$^{-1}$.

**Determination of phage titre**

The phage titre was determined by the soft agar-layer method. 0.1ml amounts of a mid log-phase culture were added to tubes containing 3ml of molten TY medium with half-strength agar (7.5g l$^{-1}$). 0.1ml of dilutions of the phage were added to separate tubes which were gently rotated to mix the contents before pouring onto prepared plates of full-strength TY agar. Plaques could be observed after incubation for two days at 30°C.

**Conjugational transfer of R factors**

Transfer of R factors was carried out using a membrane mating procedure (Jacob et al., 1976). Cultures of late log-phase bacteria, about $10^9$ v.u. ml$^{-1}$, were used in the crosses. In *Rhizobium* x *Rhizobium* crosses 0.5ml volumes of donor and recipient cultures were mixed in 5ml of TY broth. In crosses involving other species 0.1ml of donor culture was mixed with 0.5ml of the recipient culture. The suspension was passed through a Millipore membrane filter (pore size 0.45 μm, diameter 25mm), the bacteria being retained on the filter which was then transferred to the surface of a TY plate. Plates were incubated at 30°C for 20 hours. Following incubation the bacteria adhering to the filter were scraped off, resuspended in 2ml phosphate buffer, diluted and plated on appropriate selective media.

**Mapping of mutants**

The plasmid R68.45 mediates chromosomal transfer within and between several species of *Rhizobium* including *R. trifolii*
(Beringer and Hopwood, 1976; Johnston and Beringer, 1977; Beringer, Hoggan and Johnston, 1978) and was used for mapping purposes in this project.

The first step involved the construction of a suitable donor strain from that carrying the gene to be mapped, by transfer of either R68.45, or pJB3JI, a kanamycin-sensitive derivative of R68.45. A series of crosses was then carried out with nine recipient strains of *R. leguminosarum*, each carrying genetic markers, two of which flanked a section of the bacterial chromosome, the nine sections making up the whole chromosome (see Figure 2-2 and Table 2-2).

When *Rhizobium* recipients receive a section of the chromosome flanked by two genetic markers, any marker located between these will nearly always be inherited with them (Beringer, Hoggan and Johnston, 1978). It was suggested that this effect might be the result of poor recombinational ability, at least when this plasmid was used to promote chromosome transfer. Alternatively, there might be some sort of "end effect" due to preferential recombination at the ends of incoming fragments.

It was therefore possible to select for the transfer of two donor alleles e.g. *ura*⁺ and *ade*⁺ in a cross with *R. leguminosarum* 1628 as recipient and then to test the recipient bacteria for co-inheritance of the genetic marker to be mapped. The marker was then assigned to the section, which, following transfer, showed the highest proportion of recipients having that marker. A more accurate position for the marker within the section was obtained from an analysis of co-inheritance percentages with the flanking markers. The co-inheritance percentages are the frequencies with which recipients selected for one chromosomal marker also receive a second non-selected marker. Details of the crosses performed and their analyses are given in the relevant chapters.
Figure 2-2. Map of the chromosome of *Rhizobium leguminosarum* showing sections used for mapping. The strains carrying the flanking markers for each section are indicated (J.E. Beringer, personal communication).
Growth of clover plants

The plants were grown in a greenhouse with a minimum temperature of 20°C, and a 16 hour daylength. They were illuminated by banks of two warm-white and two daylight fluorescent tubes (Crompton) positioned 71cm above bench level and supplying 29,000 lux at plant height.

Preparation and inoculation of sterile clover plants

The seeds were sterilised by treating them for 15 minutes with concentrated sulphuric acid. Preliminary experiments had indicated that this was the most satisfactory treatment both in terms of sterilisation and of percentage germination (about 90%). The sterilised seeds were transferred to plates of yeast-mannitol agar since TY medium gave only low germination percentages. The plates were inverted and incubated in the dark for 2 days. Contaminated and apparently abnormal seedlings were then discarded.

The seedlings were transferred to prepared growth tubes and placed in the greenhouse for 2 days, to allow them to establish. The growth tubes were held in wooden blocks 8.7cm x 37.5cm x 4.0cm into which holes 2cm diameter by 3.2cm depth had been drilled, thus the roots obtained some degree of shading. The growth tubes were spaced 2cm apart within the rack.

Following 2 days growth the plants were inoculated by adding a suspension of the bacteria (about $10^7$ v.u. ml$^{-1}$) in liquid Jensen's medium, sufficient to cover the roots of the seedling. Plants were then grown for 5 to 6 weeks, liquid growth medium being added as required. All experiments included both uninoculated controls and nitrogen-supplied controls which contained 10mM $\text{NH}_4\text{NO}_3$ in both liquid and solid medium. Nodules began to appear approximately 10 days after inoculation, and differences between treatments, e.g. inoculated and uninoculated, generally became apparent 3 to 4 weeks after inoculation.
Preparation and sampling for the acetylene reduction assay

For acetylene reduction assays the cotton wool stopper of the test tube was replaced by a rubber Suba-seal (Gallenkamp & Co. Ltd.) and 2ml of acetylene (British Oxygen Corporation Ltd.) injected into the tube. The tubes were kept in the greenhouse for 40 hours before sampling, unless otherwise indicated. Samples of 0.2ml for assay were taken up into 1ml plastic disposable syringes (Becton, Dickinson and Company Ltd.). Where many samples were taken at one time the syringes were stuck into rubber bungs until they could be assayed. No appreciable loss of gas was observed over a period of several hours. A useful review on the preparation of material for the acetylene reduction assay, and details of the assay procedure, is given by Postgate (1972).

The acetylene reduction assay

Gas-liquid chromatography of samples was carried out on a Pye 104 gas-liquid chromatograph fitted with a 152cm column packed with Porapak R (PhaseSep Ltd., Deeside, Clwyd). The nitrogen carrier gas had a flow rate of 20ml min⁻¹, the hydrogen pressure was 1.05 kg cm⁻², and the air pressure 1.5 kg cm⁻². The column oven was held at 50°C and the detector oven at 150°C. Samples of 0.2ml were injected into the column and ethylene and acetylene gases identified by the position of their peaks on the chart recording, the peak of ethylene emerging immediately before the peak of acetylene. Ethylene was measured by a comparison of the peak height produced with that of a 0.2ml standard sample containing 9.69 nmoles of ethylene in that volume (PhaseSep Ltd.).
Chapter 3

MUTAGENESIS IN R. TRIFOLII P3
Introduction

Mutations in bacteria can occur via either of two basic routes known as repair-dependent or repair-independent. The extent to which either of these is responsible for the production of mutation in any particular species will depend on the mutagen being used, and the types of repair pathways present in the organism.

The role of repair-dependent mutation has been elucidated by work on the repair of UV-induced damage to DNA in E. coli (see reviews by Witkin, 1969, 1976; Moseley and Williams, 1977). The conclusions from such studies can be summarised as follows. UV causes the formation of pyrimidine (TT, CT, CC) dimers in DNA. These can be removed by four different mechanisms of repair (see Figure 3-1), the last three probably also being responsible for the removal of damage to DNA by other mutagenic agents.

(a) Photoreactivation repair involves the binding of a specific enzyme to a pyrimidine dimer. Following absorption of a photon of light the enzyme is able to monomerise the dimer. This process is not considered to give rise to mutations.

(b) Excision repair involves a series of enzyme-catalysed steps which result in physical removal of the damage. This pathway requires incision of the DNA adjacent to the damaged site, removal of the damage by the action of an exonuclease, DNA synthesis, to fill in the excision gap using the opposite undamaged strand as a template, and ligation of the newly synthesised DNA to the pre-existing strand. This repair mechanism is also not considered to be error-prone.

(c) Recombination repair occurs following replication of the damaged DNA. Interruption of replication opposite the damaged bases leaves gaps which are replaced by recombinational exchange with the homologous chromosome (see Figure 3-1). Where the gaps produced opposite the damaged bases following interruption
Figure 3-1. Repair of damaged DNA (based on Witkin, 1976)

Symbols
- Damaged base(s)
- Photoreactivation enzyme
- DNA polymerase
- DNA polymerised during repair synthesis
- DNA polymerised during post-mutagen treatment DNA synthesis
- Base
- Mismatched base
**PHOTOREACTIVATION** (UV DAMAGE ONLY)

Photoreactivating enzyme binds dimer

Absorption of photon. Dimer split and enzyme released.

**EXCISION REPAIR**

Endonuclease incision

Exonuclease excision and repair synthesis

Sealing by polynucleotide ligase

**RECOMBINATION REPAIR**

Replication

Recombinational exchange

Repair synthesis and sealing by ligase

**ERROR-PRONE REPAIR**

Overlapping daughter-strand gap produced following replication

Repair polymerase idles opposite damage, due to removal of mis-matched bases by proof-reading activity

Inducible function permits replication past damage. Mis-matched base(s) may be inserted

Damage still present. May be removed by excision repair or photoreactivation
of replication do not overlap in the homologous strands of DNA, repair will occur via this pathway and mutation is unlikely to occur. Where overlapping gaps are produced in this manner, a fourth error-prone process is induced (Sedgwick, 1976).

(d) Replication or error-prone repair involves an inducible function which permits replication past non-coding lesions such as pyrimidine dimers (Caillet-Fauquet, Defais and Radman, 1977), the mutagenic step arising from the random insertion of base(s) opposite (or nearby) the non-coding lesion. A model has been proposed in which the DNA polymerase "idles" opposite a non-coding lesion, due to removal of mis-matched bases by its 3' to 5' exonuclease activity (Villani, Boiteux and Radman, 1978). An induced function inhibits this "proof-reading" activity permitting polymerisation past the lesion. Because this form of repair-dependent mutagenesis is dependent on the function of the recA and lex gene products (Witkin, 1967) mutagenic agents can be classified as repair-dependent or -independent depending on their ability to induce mutation in strains of E. coli which are mutant at either of these loci (see review by Moseley and Williams, 1977).

Thus UV, gamma rays, MTC, DMTC and MMS, amongst others, are classified as repair-dependent mutagens (Witkin, 1967; Bridges, Law and Munson, 1968; Bridges et al., 1973; Kondo et al., 1970; Murayama and Otsuji, 1973).

Other mutagenic agents, classified as repair-independent, cause altered bases which mispair on replication, giving rise to mutations (see review by Drake and Baltz, 1976). Thus, nitrous acid induces deamination of cytosine to uracil and adenine to hypoxanthine generating G-T and A-C mispairs respectively. Similarly, mispairing transitions may arise due to the alkylation of bases following treatment of DNA with chemicals such as MNNG and EMS (see review by Drake and Baltz, 1976). The former is also known to react extensively with non-DNA material such as
proteins (Sugimura et al., 1968) and some experimental evidence suggests that at least a small proportion of its mutagenic effect arises from damage to enzymes involved in DNA metabolism, resulting in errors of replication (Kondo and Ichikawa, 1973). MNNG is also capable of inducing misrepair mutations (Kondo et al., 1970).

Frameshift mutations arise following additions or deletions of base-pairs in numbers not equal to multiples of three, i.e. not equal to one or more codons. These mutations are usually induced by agents capable of intercalating between adjacent base pairs, although frameshift mutations may arise via the error-prone pathway (see review by Drake and Baltz, 1976). Thus, MNNG, MMS and UV are potentially capable of inducing some frameshift mutations.

Recently, a novel method for the induction of mutations utilising transposons has been reported (Kleckner et al., 1975; Beringer et al., 1978). Transposons are discrete sequences of DNA, often coding for antibiotic resistance, capable of insertion into DNA replicons, e.g. chromosomes or plasmids, in the absence of a recA function (see review by Cohen, 1976). Insertion may be random and where it takes place in a gene, non-leaky, polar mutations arise.

Since one of the aims of this study was to isolate symbiotic mutants of *R. trifolii*, which requires screening large numbers of clones on plants to detect such mutants, it was necessary to maximise the frequency of mutants in populations of *Rhizobium*. This was done by testing various mutagenic agents for their effect on *R. trifolii* P3, as agents mutagenic for *E. coli* are not necessarily mutagenic for other bacteria. The information available from the literature does not provide any ready comparison of the data on this subject, since experiments were carried out under different conditions with different criteria of mutagen
effectiveness (see Chapter 1 for discussion of this topic). This chapter describes a comparative study of the ability of a variety of common mutagens to induce mutation to rifampicin-resistance in *R. trifolii* P3, this being chosen as a criterion of mutagen effectiveness, because mutants were readily selectable and the spontaneous mutation frequency was low.

**Methods**

Log-phase cultures, having a turbidity of between 25 and 30 units i.e. approximately $2 \times 10^8$ v.u. ml$^{-1}$, were used in all mutagenesis experiments. Measurements of viability were made during the course of each experiment. Chemical mutagens were removed by centrifugation of 0.5ml samples of the treated culture in a Quickfit microcentrifuge (Corning Ltd., Stone, Staffs.) and resuspended in TY medium, this procedure being carried out twice. In irradiation experiments, samples were diluted directly for viability measurements.

The measurement of mutation frequency was carried out by removing 0.1ml of each sample into 10ml fresh TY broth in a 100ml Erlenmeyer flask. These were incubated until turbid cultures were obtained and samples plated on selective (TY containing rifampicin, unless otherwise indicated) and non-selective media for determination of mutant and total numbers respectively. Where low numbers of mutants were anticipated, the culture was concentrated by centrifugation and resuspension in one-tenth of the original volume before plating. Calculation of mutation frequency was by dividing the number of mutants by the total number of viable units for that population. All experiments, unless otherwise indicated, were carried out with *R. trifolii* P3. The results shown in Figures 3-3 to 3-11 are each from a single representative experiments chosen from a few which were carried out.
Treatment with MNNG, EMS, MMS and DMTC

A sample of culture was centrifuged, washed and the bacteria resuspended in half the volume of phosphate buffer pH 7.0, unless otherwise stated. To this was added an equal volume of phosphate buffer containing dissolved mutagen to give the desired final concentration.

Treatment with nitrous acid

A 9ml log-phase culture was centrifuged and resuspended in 3ml distilled water. To the suspension was added 3ml acetate buffer 0.05M pH 4.5 and 3ml 0.01M NaNO₂. The mutagenic action of the nitrous acid generated was stopped by dilution of samples into TY broth for viability or determination of mutation frequency.

UV irradiation

Log-phase cultures were washed twice and resuspended in phosphate buffer. 5ml amounts were irradiated in a glass petri-dish (9cm diameter) while being agitated by means of a magnetic stirrer. The UV source was a Hanovia model 12 germicidal lamp (Hanovia Lamps Ltd., Slough, Bucks.) with an incident dose rate of 2.25 J m⁻² s⁻¹.

Gamma-ray irradiation

Log-phase bacteria in TY broth were irradiated in a ⁶⁰Co source. Oxygen was bubbled through the bacterial suspension during irradiation. The dose rate at the time of irradiation was 3.37 krad min⁻¹.

Transposon mutagenesis

Crosses were made between E. coli and appropriate recipients of R. trifolii (see Results). Selection was applied for the transfer of the transposon Tn5 (Km⁺) to the recipient. Presumptive mutants were tested for auxotrophy as described in Chapter 2.
Choice of a suitable marker for the study of induction of mutation

Rifampicin-resistance (rif) was chosen as a suitable marker for these experiments because it was readily selectable and because in *R. trifolii* P3 its spontaneous mutation frequency was fairly low, $2 \times 10^{-8}$. It was not anticipated that the results would be appreciably different for any other marker studied.

Results

Induced mutagenesis in *R. trifolii* P3

*R. trifolii* P3 was treated with a variety of mutagenic agents and induced mutation to rifampicin-resistance measured. The results are shown in Figures 3-2 to 3-9 and summarised in Table 3-1. Only two mutagenic agents were found to increase significantly the mutation frequency of this marker. The most effective was the chemical mutagen MNNG (Figure 3-2). An Experiment with this mutagen was carried out in both TY broth and phosphate buffer, at concentrations of 75 μg ml$^{-1}$. The maximum yield of mutants from the population treated in TY broth was greater than from that treated in phosphate buffer, approximately twice as many being obtained under the former conditions. The absolute mutation frequencies of rifampicin-resistant mutants for cultures treated in phosphate buffer and TY broth were $2.75 \times 10^{-6}$ and $5.5 \times 10^{-6}$ respectively.

UV was the only other mutagenic agent found to be capable of mutating *R. trifolii* P3 (Figure 3-3). A maximum frequency of $8 \times 10^{-7}$ rifampicin-resistant mutants was obtained, representing an approximately 45-fold increase over the spontaneous mutation frequency.

No evidence of any significant increase in the mutation frequency of rifampicin-resistant mutants was found using a range of other mutagenic agents (EMS, MMS, DMTC, nitrous acid and gamma rays; Figures 3-4 to 3-9). In all cases the
Figure 3-2. The effect of MNNG (75 µg ml⁻¹) on viability (closed symbols) and mutation (open symbols) of R. trifolii in phosphate buffer (●○) or TY broth (▲△)
Figure 3-3. The effect of UV irradiation on viability (●) and mutation (○) of R. trifolii P3
Figure 3-4. The effect of EMS (0.25%) on viability (●) and mutation (○) of *R. trifolii* in phosphate buffer.
Figure 3-5. The effect of EMS (0.25%) on viability (●) and mutation (○) of *R. trifolii* in Tris/saline buffer.
Figure 3-6. The effect of MMS (0.25%) on viability (●) and mutation (○) of *R. trifolii*
Figure 3-7. The effect of DMTC (0.5 μg ml$^{-1}$) on viability (●) and mutation (○) of _R. trifolii_ P3
Figure 3-8. The effect of nitrous acid on viability (●) and mutation (○) of *R. trifolii* P3.
Figure 3-9. The effect of gamma rays on survival (●) and mutation (○) of R. trifolii P3.
Table 3-I. A comparison of the lethal and mutagenic effects of a variety of common mutagenic agents in *E. coli* B/r and *R. trifolii* P3

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Lethality (D$_{37}$) (a)</th>
<th>Ratio of lethality R. trifolii P3:E. coli B/r</th>
<th>Mutagenesis (increase over spontaneous mutation frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli B/r (b)</td>
<td>R. trifolii P3</td>
<td></td>
</tr>
<tr>
<td>MNNG (TY broth)</td>
<td>-</td>
<td>29 µg ml$^{-1}$ hr</td>
<td>-</td>
</tr>
<tr>
<td>MNNG (Phosphate buffer)</td>
<td>10 µg ml$^{-1}$ hr</td>
<td>60 µg ml$^{-1}$ hr</td>
<td>6.0</td>
</tr>
<tr>
<td>UV</td>
<td>30J m$^{-2}$</td>
<td>18J m$^{-2}$</td>
<td>6.0 x 10$^{-1}$</td>
</tr>
<tr>
<td>EMS (Phosphate buffer)</td>
<td>5% hr</td>
<td>0.07% hr</td>
<td>1.4 x 10$^{-2}$</td>
</tr>
<tr>
<td>EMS (Tris-saline)</td>
<td>-</td>
<td>0.15% hr</td>
<td>-</td>
</tr>
<tr>
<td>MMS</td>
<td>0.5% hr</td>
<td>0.05% hr</td>
<td>1.0 x 10$^{-1}$</td>
</tr>
<tr>
<td>DMTC</td>
<td>-</td>
<td>0.03 µg ml$^{-1}$ hr</td>
<td>-</td>
</tr>
<tr>
<td>Nitrous acid</td>
<td>38 µg ml$^{-1}$ hr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gamma rays</td>
<td>9 krad</td>
<td>2 krad</td>
<td>2.2 x 10$^{-1}$</td>
</tr>
</tbody>
</table>

(a) A D$_{37}$ value is the dose required to reduce viability of the original population to 37% and is equivalent to that needed to kill a single bacterium. These values were calculated assuming the dose received is a function of the concentration of the mutagen used multiplied by the time of exposure.

(b) Data for *E. coli* B/r from Sweet and Moseley (1976).

(c) Mutation to trimethoprim-resistance. Spontaneous mutation frequency, $1 \times 10^{-6}$.

(d) Mutation to rifampicin-resistance. Spontaneous mutation frequency, $2 \times 10^{-8}$. 


mutagens were used at concentrations or doses which were lethal for a fraction of the population during the course of the experiment, thus it was likely that DNA damage which might be potentially mutagenic was occurring. In the case of nitrous acid, however, it was considered that some killing was simply due to the low pH at which the experiment was carried out, rather than direct damage to the DNA of the bacteria. In an experiment in which a culture of *R. trifolii* P3 was resuspended in 0.05M acetate buffer, pH 4.5, the bacteria were rapidly inactivated, 63% of the population being killed within 2 minutes exposure.

EMS mutagenesis experiments were carried out both in phosphate buffer pH 7.0, and in Tris/saline buffer pH 7.6. The latter was used in an experiment on EMS mutagenesis in *R. meliloti* 2011 in which a 300-fold increase in the mutation frequency of rifampicin-resistant mutants was observed (Meade and Signer, 1977). In neither case was any significant mutagenesis observed (Figures 3-4 and 3-5).

**EMS mutagenesis in *R. meliloti* 2011**

An EMS mutagenesis experiment of *R. meliloti* 2011, using similar conditions to those of Meade and Signer (1977) was carried out as a 'control' for the experiment described above in which *R. trifolii* was treated in the same manner. Unfortunately these workers did not state the exact concentration of EMS that they used - "two drops of ethylmethyl sulphonate were added per ml" is the only indication of quantities used. In addition, their reference to the recipe for Tris/saline buffer pH 7.6 (Miller, 1972) does not reveal specific directions for its formulation. Two separate formulations, one for saline 8.5g l⁻¹ and the other for 0.2M Tris, were therefore combined. Thus, this experiment was conducted under conditions close to but probably not identical with those used by Meade and Signer (1977).
The experiment was carried out with 1% EMS. The mutation frequency was found only to increase to a maximum of approximately 10-fold, this being at a survival value of around 2.5% (Figure 3-10). This contrasts with Meade and Signer's report of a 300-fold increase in the number of rifampicin-resistant mutants at a survival value of 25%. At a corresponding survival value in this experiment, the yield of mutants was only increased 3-fold. The spontaneous mutation frequency of rifampicin-resistant mutants was found to be ten times higher than in R. trifolii P3; thus the maximum absolute mutation frequency obtained was 2.5 x 10^-6.

EMS mutation of an auxotroph

The inability of EMS to induce mutations in R. trifolii P3 was rather surprising since this alkylating agent is normally a very efficient mutagen, as can be seen from the data on E. coli B/r in Table 3-1. Although it was considered unlikely that EMS might preferentially mutate some genes and not others, an experiment was carried out to determine the ability of this mutagen to cause reversion of an ade auxotroph of R. trifolii. As for rifampicin-resistance, no significant increase in the frequency of prototrophic revertants of R. trifolii DC13t was detected following EMS treatment (Figure 3-11).

EMS mutagenesis of other strains of R. trifolii

A plate test was devised in which late log-phase cultures were plated onto SY plates and allowed to dry. A 10 μl drop of mutagen, 100% EMS, was placed on the centre of the plates which were then incubated overnight at 22°C. A 3ml layer of half-strength SY medium containing rifampicin sufficient to give the desired final concentration was then poured onto the plates which were incubated for another 5 days at 30°C. Total counts were also obtained for each culture plated. The results of this experiment are presented in Table 3-2.
Figure 3-10. The effect of EMS (1%) on survival (*) and mutation (○) of *R. meliloti* 2011
Figure 3-11. The effect of EMS (0.25%) on viability (●) and reversion to prototrophy (○) of an adenine auxotroph of R. trifolii P3
### Table 3-2

The mutagenic effect of EMS on a variety of strains of *R. trifolii* as detected in a plate test.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spontaneous</th>
<th>EMS-induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>$2 \times 10^{-8}$</td>
<td>$3 \times 10^{-8}$</td>
</tr>
<tr>
<td>RCR1</td>
<td>$&lt;1 \times 10^{-7}$</td>
<td>$2 \times 10^{-7}$</td>
</tr>
<tr>
<td>1DL</td>
<td>$&lt;2 \times 10^{-8}$</td>
<td>$7 \times 10^{-8}$</td>
</tr>
<tr>
<td>RCR32</td>
<td>$5 \times 10^{-8}$</td>
<td>$3 \times 10^{-7}$</td>
</tr>
<tr>
<td>RCR5</td>
<td>$2 \times 10^{-7}$</td>
<td>$3 \times 10^{-7}$</td>
</tr>
<tr>
<td>RCR4</td>
<td>$5 \times 10^{-8}$</td>
<td>$5 \times 10^{-8}$</td>
</tr>
<tr>
<td>RCR49</td>
<td>$5 \times 10^{-7}$</td>
<td>$6 \times 10^{-7}$</td>
</tr>
<tr>
<td>FA6</td>
<td>$3 \times 10^{-7}$</td>
<td>$4 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
The results show that there are indeed strain variations in response to EMS mutagenesis. RCR32 shows an almost 5-fold increase in the number of mutants following this treatment. Strains RCR1, 1DL and RCR49 may also be mutated by EMS, although the extent to which this occurred could not be calculated since accurate values for the spontaneous mutation frequencies were not obtained. Although these results would require confirmation in experiments such as described previously, it would appear unlikely that any of these strains would be mutagenised significantly more than R. trifolii P3.

**Tn5 mutagenesis**

The plasmid pJB4J1 carries the transposon Tn5 which codes for kanamycin resistance. It is unstable in *Rhizobium* spp. because of the presence of phage Mu which is also inserted in this plasmid (Boucher *et al.*, 1977; Beringer *et al.*, 1978). Thus, kanamycin-resistance in rhizobia derived from the transfer of this plasmid from *E. coli* will almost certainly have the transposon inserted into either their chromosomal or plasmid DNA, although in a few cases pJB4J1 may be stabilised in the recipient *Rhizobium* due to large deletions of the Mu phage (van Vliet *et al.*, 1978). The kanamycin-resistant recipients can then be tested for the desired mutation.

To investigate the induction of rifampicin-resistant mutants, a cross was carried out between *E. coli* 1830 and *R. trifolii* DC2t. The exconjugants from this experiment were resuspended in 0.5ml buffer. The suspension was plated for selection of kanamycin transfer and also simultaneously for kanamycin transfer and mutation to rifampicin resistance. It contained $4.8 \times 10^4$ kanamycin-resistant recipients ml$^{-1}$ but no rifampicin-resistant mutants were detected. The reasons for this are discussed fully in a later section.
It was decided, therefore, to compare the mutagenesis of auxotrophs by this method with that by MNNG. An MNNG-treated culture which yielded a frequency of $3 \times 10^{-6}$ rifampicin-resistant mutants was screened for the presence of auxotrophs. Out of 2,718 colonies examined, eight auxotrophs were isolated, a frequency of 0.3%. The requirements of two of these were identified, *R. trifolii* DC10t being capable of growth on methionine and *R. trifolii* DC13t capable of growth on either adenine or hypoxanthine. In another, similar, experiment a further three auxotrophic strains were identified, *R. trifolii* DC9t, DC11t and DC12t, these being cysteine-, methionine- and tryptophan-requiring respectively.

In a cross between *E. coli* 1830 and *R. trifolii* DC1t in which the frequency of transfer of kanamycin-resistance per recipient was $3.5 \times 10^{-6}$, a total of 4,600 colonies were screened for auxotrophy. Thirteen auxotrophs representing a frequency of 0.3% were isolated. Two were found to require histidine, two methionine, two adenine or hypoxanthine, one uracil, one cysteine, one tryptophan, and four could not be identified. Since isolates with the same requirement could not be guaranteed as independent strains, only one isolate of each type was purified for use as a stock strain (see Table 2-1).

The results show that the frequency of auxotrophs induced by either of these methods is very similar. The advantage of transposon mutagenesis, however, was that the nutritional requirements of a greater proportion of the auxotrophs could be identified - eight out of thirteen, as opposed to two out of eight following MNNG mutagenesis. This is presumably because the latter treatment is generating a relatively high number of doubly or multiply auxotrophic mutants due to its localised action at the replication fork (Guerola, Ingraham and Cerdá-Olmedo, 1971), these not being readily identifiable.
The range of auxotrophs found which includes all of the types generated by MNNG mutagenesis suggests that insertion of the transposon is fairly random, at least with regard to the chromosome as a whole, although preferred sites within short regions of DNA are likely to exist (Botstein and Kleckner, 1977).

Discussion

The induction of mutations in *R. trifolii*

The ability of only two out of a variety of common mutagens to cause mutation in *R. trifolii* P3, i.e. MNNG and UV, confirmed one of the reasons for carrying out this study, namely that agents mutagenic for *E. coli* are not necessarily mutagenic for other organisms. MNNG was found to be the most effective mutagen and indeed has often been the agent of choice for the generation of mutants for genetic studies in other species of *Rhizobium* (Kondorosi et al., 1977a, b; Ludwig and Signer, 1977; Beringer, Hoggan and Johnston, 1978; Casadesús and Olivares, 1979).

The inability of EMS to generate mutations in *R. trifolii* P3 either forward, to rifampicin resistance, or back, to prototrophy, was rather surprising since it is a very efficient mutagen for *E. coli* B/r and has also been reported to be effective in *R. meliloti* 2011, causing a 300-fold increase in the number of rif mutants (Meade and Signer, 1977). Under conditions similar to those used by Meade and Signer (1977) there was still no evidence of mutagenesis in *R. trifolii* P3 and only a 10-fold increase in the number of rif mutants of *R. meliloti* was observed. The reason for the discrepancy between the latter result and that obtained by Meade and Signer (1977) is not clear. The fact that the conditions were not absolutely identical in these two experiments (see Results section) might provide a partial explanation. Alternatively, although the two strains of *R. meliloti* used were
nominally the same, it is likely that some time has elapsed since they were originally taken as clones from the same parental strain. It is possible that during this time they have diverged, such that many of their characteristics are no longer identical. Indeed, an experiment to test the response of some different strains of _R. trifolii_ to EMS, indicated that there are differences in their responses to mutagenesis.

The practical conclusions to be drawn from the work described in this chapter are that in initiating genetic studies which require the isolation of mutants, particularly non-selectable mutants, it is a worthwhile exercise to investigate the efficacy of a variety of mutagens. Another possibility worth examining is the variability of response of different strains to some mutagens, since some may respond better than others, although no significant differences were observed in this study. In addition, the conditions under which the mutagenesis experiment is carried out may alter the result, e.g. MNNG was found to yield twice as many mutants of _R. trifolii_ when the bacteria were treated in TY broth as opposed to phosphate buffer. Other variables such as pH, ionic strength or composition of the buffer used might also affect the outcome of a mutagenesis experiment. These particular possibilities were not examined in this work.

**Repair and mutagenesis in _R. trifolii_ P3**

The extent of mutation induced by UV and MNNG in _R. trifolii_ P3 is relatively low when compared with the maximum possible induced level for _E. coli_ B/r, i.e. a 1000-fold increase over the spontaneous mutation frequency for trimethoprim-resistance for EMS mutagenesis (see Table 3-1). The reasons for this resistance to mutagenesis are not clear. As mentioned in the introduction to this chapter, the mechanism of mutation, i.e. repair-dependent or -independent, and the pathways of repair present in the organism will influence the extent of induced mutation.
The induction of mutation by UV strongly suggests that R. trifolii P3 possesses an error-prone pathway, although other mutagens such as MMS, DMTC and gamma-rays which operate via this pathway to a lesser extent were not mutagenic. No evidence was found of induction of mutation by two repair-independent mutagens capable of causing mispairing, i.e. EMS and nitrous acid. MNNG may act both in a repair-independent and -dependent manner in addition to its effects on enzymes involved in DNA metabolism, therefore its mode of action in the induction of mutations in R. trifolii may involve all or some of these mechanisms.

The repair of DNA damage caused by some mutagens, e.g. MNNG, UV and gamma-rays is approximately as efficient in R. trifolii P3 as in E. coli B/r, and the first two of these are mutagenic for R. trifolii P3 (see Table 3-1). Gamma-rays were not mutagenic for R. trifolii P3, but in any case were only weakly mutagenic for E. coli B/r. Damage caused by MMS and particularly EMS was not dealt with as efficiently by R. trifolii P3, and no induction of mutations was detected in this organism. This suggests that the sensitivity of R. trifolii to these mutagens was such that lethal events were more likely to occur in this organism than mutagenic events when compared with the same damage in E. coli. In addition, the greater sensitivity of R. trifolii P3 meant that surviving populations in which any induced mutations might be detected would be smaller and therefore the chances of detecting such mutations would also be decreased.

In the case of DMTC it is possible that no repair of damage occurs at all. Assuming that the rate of formation of adducts by DMTC is approximately the same as that for MTC as calculated by Moseley and Copland (1975), and that the molecular weight of the R. trifolii chromosome is similar to that of E. coli,
then the $D_{37}$ value of 0.03 $\mu g \text{ ml}^{-1} \text{ hr}$ for *R. trifoliium* indicates that a single adduct is lethal for this organism.

**Transposon mutagenesis**

The kinetics of transposition mutagenesis are such that it is not possible to readily detect a mutation in a specific gene in the recipients of a single cross. This was confirmed in an experiment to detect the induction of *rif* mutants by this method, none being found.

The yield of recipients carrying Tn5(Km $^R$) from a cross is approximately $3 \times 10^3$. Assuming that the genome of *R. trifoliium* has approximately the same number of genes as *E. coli*, i.e. about 3,000 (see Watson, 1970), then the probability of insertion within a particular gene is about $3 \times 10^{-4}$. Thus, even if all the recipients from a single cross had arisen from independent insertion events, which is unlikely, the chance of obtaining a particular mutant will be low, i.e. a maximum probability of $10^{-1}$ for a single cross.

Where the type of mutation sought may result from insertion in any of a number of genes, e.g. mutation to auxotrophy, then it should be possible to detect such in a single cross. Thus, a yield of 0.3% auxotrophs was induced by this method. The range of auxotrophs isolated was similar to that produced by MNNG mutagenesis. No steps were taken in this study to confirm that the auxotrophs isolated by the former method resulted from insertion of Tn5, e.g. by conjugational transfer of the kanamycin marker and analysis of the coinheritance frequency of the auxotrophic marker which should be 100%. It is likely, however, that in most, if not all, isolates insertion of Tn5 was responsible for the phenotype produced. Beringer *et al.* (1978) found that for three auxotrophic mutants of *R. leguminosarum* isolated following Tn5 mutagenesis, cotransduction frequencies of these markers with kanamycin-resistance were 100%.
Practical implications

The maximum possible induced mutation frequency obtained by MNNG mutagenesis was still low for the purposes of mutant screening, e.g. for the isolation of a specific symbiotic mutant such as a derepressed mutant. A number of approaches to enhance the "mutability" of R. trifolii were taken, including the isolation of a UV-sensitive mutant (see Chapter 5). The isolation procedure required the use of a phage infective for this bacterium. Chapter 4 describes the isolation and some properties of a rhizobiophage.
Chapter 4

ISOLATION AND CHARACTERISATION
OF A RHIZOBIOPHAGE
Introduction

Rhizobiophages are readily isolated from the roots and nodules of naturally-grown leguminous plants or from soil in which such plants are found, but are seldom found in soil which has not supported the growth of these plants (Kleczkowska, 1957). More than 100 isolates of such bacteriophages have been described in the literature (see review by Ackermann, 1978), although many of these are almost certainly identical. These isolates include both lytic (Kleczkowska, 1957; Atkins, 1973; Buchanan-Wollaston, 1979) and temperate phages (Marshall, 1956; Takahashi and Quadling, 1961; Kowalski, 1970).

The classification of the rhizobiophages has been based almost entirely on their morphological appearance as determined by electron microscopy (Barnet, 1972; Atkins, 1973; see also review by Ackermann, 1978) although in some cases analysis of the nature (i.e. RNA or DNA), molecular weight and GC content of the nucleic acid has also contributed information (Mayer, Lotz and Lang, 1973; Atkins and Avery, 1974; Lurz, Mayer and Lotz, 1975). Biological characteristics such as burst size, latent period and resistance to physical and chemical agents, have not been found to be useful for the purpose of classification.

The study of bacteriophages infective for Rhizobium is carried out for a number of reasons. Firstly, the information acquired is useful in the general field of phage research. Secondly, rhizobiophages may have an ecological effect on the numbers and distribution of Rhizobium spp. within the soil. Thirdly, it has been proposed that phages might be used for the typing of strains of Rhizobium, as has been carried out with some other bacterial species (Staniewski, 1970). The most useful application of the study of the rhizobiophages is likely to be in the field of genetics whereby transducing phages can be used in the manipulation of chromosomal or plasmid DNA for such purposes as genetic
mapping (Buchanan-Wollaston, 1979; Casadesús and Olivares, 1979).

The rhizobiophage Rtl was isolated with a view to its use as a semi-selective agent for the isolation of a UV-sensitive mutant of R. trifolii (see Chapter 5). The opportunity was taken, however, to examine some characteristics of this virus. These are described in this chapter.

Materials and Methods
(a) Isolation of the rhizobiophage Rtl

Soil samples of approximately 5g were taken from a variety of sites around Edinburgh where clover was found to be growing. These were shaken up on 20ml phosphate buffer and allowed to stand overnight to allow the soil particles to settle. The supernatant of each sample was filtered through a Millipore filter (0.45 μm pore size) and 0.5ml of the filtrate added to 10ml of an exponential culture of R. trifolii P3. The cultures were incubated overnight, centrifuged and the supernatant sterilised as before. The filtrate was plated by the agar-layer method. Some of the samples produced plaques after incubation for 2 days at 30°C. Purified lysates were obtained by stabbing the plaques with a sterile wire, shaking them in 1ml broth, then replating as above to obtain isolated plaques. This procedure was repeated twice. No obvious differences were observed in the plaques obtained from the different phage isolates, so one (Rtl) was chosen for further study.

(b) Electron microscopy of Rtl

Drops of high titre lysate (>10^{10} p.f.u. ml^{-1}) were placed on plastic-coated grids and negatively stained with 2% aqueous uranyl acetate. The preparations were examined using a Philips 300 transmission electron microscope at an accelerating voltage of 80kV.
Results and Discussion

Description

(a) Plaque morphology

Plaques produced by phage Pt1 had a clear central zone with a diameter of about 1 mm surrounded by a more turbid zone extending 3 mm from the centre. This latter zone may be the result of diffusion of a polysaccharide depolymerising enzyme causing lysis of some bacteria surrounding the plaque (Barnet and Humphrey, 1975).

(b) Bacteriophage morphology

Plates 4-1 and 4-2 are electron micrographs of the phage at two different magnifications. The phage has an icosahedral head, a contractile tail (an example of a phage with a contracted tail can be seen in Plate 4-1 as indicated by the arrow) and a base plate, which may be a double structure having at least three attached spikes (see arrow on Plate 4-2). The dimensions and morphological characteristics of this rhizobiophage are compared with some other isolates in Table 4-1. From an examination of the information given, it can be seen that Pt1 most closely resembles phages C and I, both in terms of size and morphology. These latter phages were designated group I by Atkins (1973), and are reported to produce plaques with a turbid halo, as does Pt1. Phages C and I were observed to have fibres attached to the base plate, the absence of these in Pt1 may have been due to their loss during preparation for microscopy.

Phages C and I have unfortunately not been classified within the framework of species proposed by Ackermann (1978). It is possible that these viruses and Pt1 are strain variations of either of the species defined by the 'types' WTI and CT4, or alternatively, that they represent one or more new species. More information from serological tests, and examination of various
Plate 4-1. Electron micrograph of R1 showing phage with contracted tail (indicated by arrow). Bar represents 100nm.
Plate 4-2. Electron micrograph of Rtl. Arrow indicates phage with base plate having at least three spikes. Bar represents 100nm.
<table>
<thead>
<tr>
<th>Phage strain designation</th>
<th>Morphological group (a)</th>
<th>Capsid diameter (nm)</th>
<th>Tail length (nm)</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rt1</td>
<td>A1</td>
<td>69 (b)</td>
<td>92 (b)</td>
<td>Base plate at least 3 spikes. No evidence of fibres.</td>
<td>This thesis</td>
</tr>
<tr>
<td>c, 1</td>
<td>A1</td>
<td>74</td>
<td>115</td>
<td>At least 3 spikes on base plate. Fibres attached.</td>
<td>Atkins (1973)</td>
</tr>
<tr>
<td>m</td>
<td>A1</td>
<td>110</td>
<td>144</td>
<td>Complex structure at end of tail.</td>
<td>Atkins (1973)</td>
</tr>
<tr>
<td>e</td>
<td>A1</td>
<td>82</td>
<td>124</td>
<td>At least 5 spikes on base plate. Attached fibres terminate in spherical bodies.</td>
<td>Atkins (1973)</td>
</tr>
<tr>
<td>WT1</td>
<td>A1</td>
<td>64</td>
<td>128</td>
<td>5 spikes on base plate</td>
<td>Barnet (1972)</td>
</tr>
<tr>
<td>CT4</td>
<td>A1</td>
<td>60</td>
<td>124</td>
<td>Very small base plate with few short fibres attached.</td>
<td>Barnet (1972)</td>
</tr>
</tbody>
</table>

(a) All rhizobiophage having contractile tails are classified in morphological group A1 (Ackermann, 1978).

(b) Average of four measurements taken from Plate 4-1.

(c) These four strains are species "types" proposed by Ackermann (1978).
properties of their nucleic acids, would be useful in clarifying this issue.

**Rate of adsorption of Rtl**

Prior to carrying out a one-step growth curve of Rtl, it was necessary to determine the rate of adsorption of the phage to the bacteria. One ml of a phage lysate containing \(7.8 \times 10^9\) p.f.u. ml\(^{-1}\) was added to 9 ml of a log-phase culture of *R. trifolii* P3 having \(2.8 \times 10^8\) v.u. ml\(^{-1}\). Samples were removed at various time intervals and diluted 100-fold in broth to prevent further adsorption. These were then spun down to remove the bacteria and the supernatant was assayed for unadsorbed phage. The results of this experiment are shown in Figure 4-1. The adsorption constant (K) was calculated from the formula given below (see Adams, 1959):

\[
K = \frac{2.3 \times \log \frac{P_0}{P_t}}{(B/t)}
\]

- \(B\) = bacteria (v.u. ml\(^{-1}\)) = \(2.5 \times 10^8\)
- \(t\) = time (minutes) = 80
- \(P_0\) = phage assay at time 0 = \(7.8 \times 10^8\)
- \(P_t\) = unadsorbed phage at time \(t\) = \(1.9 \times 10^8\)

Substituting the above values in the equation gives an adsorption constant of \(7.0 \times 10^{-11}\) ml min\(^{-1}\). This is slightly lower than the range of adsorption constants for four other rhizobiophages (\(9.2 \times 10^{-11}\) to \(2.9 \times 10^{-9}\) ml min\(^{-1}\); Atkins, 1973).

**One step growth curve**

A phage lysate of \(1.2 \times 10^9\) p.f.u. ml\(^{-1}\) was diluted 10-fold into a log-phase culture of *R. trifolii* P3 (\(2.5 \times 10^8\) v.u. ml\(^{-1}\)) and adsorption allowed to occur for 2 minutes at 30°C, giving a multiplicity of infection of about 0.2. The suspension was then diluted 100-fold into pre-warmed broth to prevent further phage adsorption. The bacteria were spun down and gently resuspended in fresh pre-warmed broth to remove unadsorbed phage. This
Figure 4-1. Adsorption of phage Rt1 to *R. trifolii* P3

Figure 4-2. One-step growth curve of phage Rt1
incubation mixture was placed in a reciprocal shaking water bath and samples removed and assayed at various time intervals.

From Figure 4-2, the latent period was found to be 190 minutes, the rise period 85 minutes and the burst size was calculated to be 17. The latent period was somewhat longer than was found by Atkins (1973) for four of his phage isolates where the times ranged from 100 to 120 minutes. The rise period of 85 minutes was more or less consistent with those found by Atkins (1973) which ranged from 80 to 180 minutes. The burst size calculated was surprisingly small, sizes of 120-210 having been found previously (Atkins, 1973), but was consistent with indications from a previous trial run experiment. The number of unadsorbed phage in the discarded supernatant (following the 100-fold dilution) was assayed and found to be consistent with removal of the majority of the unadsorbed phage from the incubation mixture (data not shown). Thus it was considered unlikely that a 'false' value for the original number of uninfected bacteria was obtained, which would affect the calculation of burst size.

Host range of Rtl

A phage lysate prepared on R. trifolii P3 was plated by the agar-layer method on lawns of a variety of species and strains of Rhizobium, and the results are shown in Table 4-2.

The phage appeared to be either capable of infection at efficiencies of plating similar to that of the strain on which it was isolated, or was not capable of infection at all. Restriction and modification of rhizobiophage has been reported but the reduction of efficiency of plating was found to vary from $10^{-1}$ to $5 \times 10^{-4}$, depending on the history of the phage lysate and the strain of host Rhizobium being tested, although in a few cases this may have been as low as $10^{-6}$ (Schwinghamer, 1965). In all cases where the phage Rtl appeared to be incapable of producing plaques on the host strain the efficiency of plating was less than $10^{-7}$. It seemed
Table 4-2. Host range of Rti

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Efficiency of plating (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. trifolii</td>
<td>P3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1DL</td>
<td>8.6 x 10^{-1}</td>
</tr>
<tr>
<td></td>
<td>RCR32</td>
<td>8.0 x 10^{-1}</td>
</tr>
<tr>
<td></td>
<td>FA6</td>
<td>&lt; 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>RCR1</td>
<td>&lt; 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>RCR4</td>
<td>&lt; 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>RCR5</td>
<td>&lt; 10^{-7}</td>
</tr>
<tr>
<td></td>
<td>RCR49</td>
<td>&lt; 10^{-7}</td>
</tr>
<tr>
<td>R. leguminosarum</td>
<td>300</td>
<td>&lt; 10^{-7}</td>
</tr>
<tr>
<td>R. phaseoli</td>
<td>8086</td>
<td>7.8 x 10^{-1}</td>
</tr>
<tr>
<td>R. meliloti</td>
<td>2001</td>
<td>&lt; 10^{-7}</td>
</tr>
</tbody>
</table>

(a) Relative to host strain of R. trifolii P3.

Table 4-3. Stability of Rtl in a number of diluents

<table>
<thead>
<tr>
<th>Solution</th>
<th>Original phage titre (p.f.u. ml^{-1})</th>
<th>Phage titre after 30 min at 30°C (p.f.u. ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>TY broth</td>
<td>1.75 x 10^8</td>
<td>1.6 x 10^8</td>
</tr>
<tr>
<td>Phosphate buffer (0.067M)</td>
<td>1.75 x 10^8</td>
<td>&lt; 10^5</td>
</tr>
<tr>
<td>SSC</td>
<td>1.75 x 10^8</td>
<td>&lt; 10^5</td>
</tr>
<tr>
<td>Phage buffer</td>
<td>1.75 x 10^8</td>
<td>&lt; 10^5</td>
</tr>
<tr>
<td>Phosphate buffer + 0.1% glycine</td>
<td>1.10 x 10^8</td>
<td>&lt; 10^5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.10 x 10^8</td>
<td>1.2 x 10^8</td>
</tr>
</tbody>
</table>
unlikely, therefore, that host restriction and modification was responsible for the inability of the phage to infect some strains. It is possible that these simply did not possess receptor sites for this virus.

**Stability of Rtl in some diluents**

It was observed during the course of this work that Rtl was unstable in certain buffer solutions. In order to determine the UV-sensitivity of the phage (see below) it was necessary to suspend it in a UV-"transparent" solution. A number of possible diluents were therefore investigated for the stability of Rtl when suspended in them. This was carried out by diluting a lysate of approximately $10^{10}$ p.f.u. ml$^{-1}$ a 100-fold into the solution to be tested. These had previously been equilibrated at 30°C and were incubated a further 30 minutes before assaying for phage titre. The results are presented in Table 4-3.

The phage was unstable in a variety of buffers. Instability such as this has been found in some instances to be due to inactivation of the virus at gas-liquid interfaces, an effect which can be reduced by the addition of protein (see Adams, 1959). The addition of glycine to phosphate buffer had no effect on the stability of the phage which suggested that this was not the mechanism of inactivation. Also, Rtl was found to be stable in distilled water. It is likely, therefore, that one or more of the salts present in the three buffers was affecting the stability of the virus. No steps were taken to ascertain which of the chemicals used were responsible.

Although Rtl was found to be quite stable in distilled water over short periods of time, the titre was observed to decline slowly over longer periods of time, i.e. days, whereas the titre in TY broth remained constant.

**The effect of temperature on Rtl**

A suspension of the phage was diluted 100-fold into broth previously equilibrated at the temperature to be tested. The
mixtures were then incubated for 30 minutes before assaying for phage titre. The results shown in Figure 4-3 indicate that the phage is rapidly inactivated at temperatures of around 50°C and greater. The temperature coefficient of inactivation for phages has been found to be consistent with that for the heat denaturation of proteins (see Adams, 1959). The inactivation of the phage is therefore due to breakdown of its protein coat. For practical purposes, this experiment has shown that it is important not to allow the temperature of the molten agar used in the agar-layer method to rise much above the minimum 42°C required. It also shows the impracticality of a heat-treatment method for sterilisation of lysates as is sometimes employed.

**The effect of ultraviolet light on Rtl**

A high-titre lysate of Rtl was diluted a 100-fold into distilled water and a 5ml amount was irradiated as described in Chapter 3. Samples were tested for phage titre at various time intervals. The results shown in Figure 4-4 indicate a $D_{37}$ value for phage inactivation of 54J m$^{-2}$. Having determined the UV-sensitivity of Rtl, it was possible to use the irradiated virus in an experiment to isolate a UV-sensitive mutant of *R. trifolii* P3 (see Chapter 5).

**Is Rtl a transducing phage?**

Transduction of the rif (rifampicin-resistance) and str (streptomycin-resistance) markers from *R. trifolii* DC4t was attempted. These genes are closely linked in *R. leguminosarum* (Beringer, Hoggan and Johnston, 1977) and evidence from an experiment on conjugal transfer of markers suggested that this was also true for *R. trifolii* (see Chapter 5). An experiment was therefore carried out to detect if phage Rtl was capable of transducing rif and str markers either together or singly. Even if transduction was at a very low frequency it might be detected where transfer of both rif and str were selected simultaneously,
Figure 4-3. The effect of temperature on phage Rt1
Figure 4-4. The effect of UV irradiation on survival of phage Rt1
since the appearance of even a very low number of transductants would still be considerably greater than the probability of obtaining such a double mutant.

The method, which was based on that of Buchanan-Wollaston (1979), involved the production of a high titre lysate on \( \text{R. trifolii} \) DC4t by the agar-layer method. The lysate (5 x \( 10^8 \) p.f.u. ml\(^{-1}\)) was then irradiated with UV (225J m\(^{-2}\)), reducing the titre about \( 10^{-2} \) before mixing with an equal volume of late log-phase bacteria (about \( 10^9 \) v.u. ml\(^{-1}\)). The suspension was incubated for 150 minutes at \( 30^\circ \)C, plated onto SY agar and incubated overnight at \( 30^\circ \)C. The plates were then overlaid with 3ml half-strength SY agar medium containing the appropriate amounts of antibiotics to give the required final concentrations. Selection was made with rifampicin and streptomycin both singly and together. The plates were incubated a further 5 days before examination.

No evidence of transduction was found in an experiment where the lower limit of transduction which could be detected was \( 5 \times 10^{-8} \) per recipient. It would therefore seem, at least on the basis of this preliminary experiment, that Rtl is not a transducing phage.

**Summary**

A phage infective on \( \text{R. trifolii} \) P3 was isolated. Electron microscopy of a phage lysate revealed that the phage had an icosahedral head and contractile tail, thus placing it in the morphological group A1 according to Ackermann (1978). Measurements of its dimensions, in addition to its morphological appearance, suggested it possibly belonged to one or other of the species defined by the "types" WT1 or CT4 (Ackermann, 1978). Following determination of the rate of adsorption of Rtl to \( \text{R. trifolii} \) P3, a one-step growth curve was carried out. Measurements of the latent period and rise period were consistent with those for other rhizobiophage but the burst size was small.
The phage was infective for two other strains of *R. trifolii* and one strain of *R. phaseoli*.

Rtl was unstable at temperatures of 50°C and above. It was also rapidly inactivated in some buffers, although it was relatively stable in distilled water. The effect of ultraviolet light on Rtl was determined and a $D_{37}$ of 54J m$^{-2}$ calculated. Preliminary evidence has indicated that Rtl is not a transducing phage.
Chapter 5

ATTEMPTS TO ENHANCE MUTAGENESIS IN R. TRIFOLII P3
Introduction

Enhancement of mutagenesis in bacteria may be brought about in three different ways. Firstly, chemical treatment of the bacteria immediately following mutagenesis may increase mutation yields. Secondly, mutants may be isolated which are more responsive to one or more specific mutagens. Lastly, certain plasmids enhance mutagenesis in their host strain. These three possibilities are discussed below.

Post-mutagenesis treatment

Treatment of UV-irradiated E. coli with caffeine enhances the induction of mutations (Sideropoulos and Shankel, 1968). This has been attributed to the binding of this chemical to the uvrA, B endonuclease, thus inhibiting the removal of pyrimidine dimers by excision repair, and increasing their probability of being dealt with by the error-prone pathway (Braun and Grossman, 1974). Caffeine also further enhances UV mutagenesis in Salmonella typhimurium harbouring a plasmid which increased UV mutability (MacPhee, 1973). Acriflavine and other basic dyes, as well as caffeine, can enhance UV mutagenesis (Witkin, 1961).

Mutants with increased susceptibility to mutagenesis

A mutant of Micrococcus radiodurans, sensitive to mitomycin C, has increased susceptibility to mutagenesis by MMS, EMS and MNNG, but is not correspondingly susceptible to their lethal effects (Moseley and Copland, 1978; Tempest, 1978). It is between 100 and 400 times more mutable by EMS and MMS and 50 times more mutable by MNNG. The lesion resulting in this phenotype has been shown to reside in one gene designated mtcA (Moseley and Copland, 1978). The nature of the defect is not clear but evidence suggests that it may lack a correctional endonuclease or a glycosylase, i.e. an enzyme which specifically removes a damaged base by hydrolysing the bond between it and
the deoxyribose moiety (Tempest, 1978).

Some UV-sensitive mutants of *E. coli* are more susceptible to the mutagenic effects of ultraviolet light, mutations being produced at higher frequencies at low doses of UV (Witkin, 1966, 1967). These *uvr* strains lack an endonuclease involved in the excision repair process (Braun and Grossman, 1974) so that a greater proportion of the thymine dimers induced by UV are "channelled" through the error-prone pathway, resulting in an enhanced yield of mutants even at low UV doses.

**Plasmid enhancement of mutagenesis**

A number of plasmids increase the mutagenic effect of UV and reduce its lethal effect. These include *R* factors (Drabble and Stocker, 1968; Lehrbach, Kung and Lee, 1978), *FP* sex factors (Krishnapillai, 1975) and *Col* factors (Howarth, 1965; Siccardi, 1969) and their effects have been observed in *Salmonella typhimurium* (Mortelmans and Stocker, 1976), *Pseudomonas aeruginosa* (Lehrbach, Kung and Lee, 1978) and *E. coli* (Siccardi, 1969; Oliver and Stacey, 1977). Enhancement of mutation by a number of chemical mutagens such as MMS, MNNG and EMS has also been observed (McCann *et al.*, 1975; Venturini and Monti-Bragadin, 1978).

The manner in which these plasmids produce their effects has not been completely elucidated. They are capable of operating in *uvr* and *polA* strains but not *recA* strains (Mortelmans and Stocker, 1976; Lehrbach *et al.*, 1977; Walker, 1977; Kung and Lee, 1978). This has led to the suggestion that these plasmids may in some manner contribute to, and thereby amplify, the effects of the error-prone pathway of repair (Krishnapillai, 1975; Mortelmans and Stocker, 1976; Walker and Dobson, 1979). Evidence suggests that the contributed function may be a polymerase for the plasmids pMG2 (Lehrbach *et al.*, 1977; Lehrbach, Kung and Lee, 1977) and *R-Utrecht* (MacPhee, 1974).
Aim of the work described in this chapter

As noted in Chapter 3, even the most efficient mutagen for *R. trifolii* P3, i.e. MNNG, only increased the mutation frequency 400-fold to a maximum of $5.5 \times 10^{-6}$. In terms of screening clones on plants for specific mutations, e.g. derepression of nitrogen fixation, this frequency of induced mutation is unacceptably low. This chapter describes a number of attempts, based on some of the approaches described above, to increase the responsiveness of *R. trifolii* P3 to at least some mutagens.

Materials and Methods

**Caffeine enhancement of mutation by UV**

A log-phase culture of *R. trifolii* P3 (about $2 \times 10^9$ v.u. ml$^{-1}$) was exposed to $45J m^{-2}$ UV. 0.1ml samples were then added to 10ml amounts of TY broth each containing different concentrations of dissolved caffeine. These cultures were incubated until turbid (2-3 days), then plated for measurement of mutation frequency.

**Mitomycin C sensitivity of *R. trifolii* P3**

A log-phase culture of *R. trifolii* P3 was washed twice, resuspended in half its volume of phosphate buffer which was then added to an equal volume of phosphate buffer containing mitomycin C dissolved at a concentration of 5 µg ml$^{-1}$. Samples were plated for viable counts at appropriate time intervals.

**Isolation of a UV-sensitive mutant of *R. trifolii* P3**

The semi-selective isolation method used was based on that of Howard-Flanders and Theriot (1962). A high titre lysate of phage Rt1 was spun down at 80,000g for 2 hours and resuspended in an equal volume of distilled water ($1.3 \times 10^{10}$ p.f.u. ml$^{-1}$). The lysate was exposed to a UV dose of 157.5J m$^{-2}$ (13% survival)
then equilibrated at 30°C before adding to an equal volume of log-phase bacteria (7.0 x 10^7 v.u. ml⁻¹). Adsorption was allowed to take place for 25 minutes giving an approximate multiplicity of infection of 10 (i.e. for viable and non-viable phage), and about 1,000 bacteria were spread per plate. These were incubated for 3 days and surviving colonies were streaked across TY plates and successive sections were exposed to 0, 45, 90 and 135 J m⁻² UV.

UV and gamma-ray irradiation and treatment with MMS

These experiments were carried out as described previously (see Chapter 4). For some experiments with the UV-sensitive mutant, the dose rate of the lamp was reduced by wrapping it in silver foil, apart from a 2 cm centre section. In these cases the dose rate was calculated either from a knowledge of the D₃⁷ of wild-type or the UV-sensitive mutant itself, as indicated in the Results section.

Mapping of the uvs marker

Mapping was carried out as described in Chapter 2. For these experiments adenine was used at a concentration of 40 μg ml⁻¹.

Conjugational transfer of plasmids

These experiments were carried out as described in Chapter 2. In E. coli x E. coli and Pseudomonas aeruginosa x E. coli 0.5 ml volumes of donor and recipient were used. Where R. trifolii was the recipient involving either of these species as donor, 0.1 ml volumes of the latter were used.

Transformation: Method 1

(a) Preparation of plasmid DNA

The method was based on that of Humphreys, Willshaw and Anderson (1975). One litre of E. coli 24-2 was grown in M9 medium supplemented with threonine and leucine to a density of about 10⁹ v.u. ml⁻¹. The cells were harvested by centrifugation at 10,000 r.p.m. at 4°C for 15 minutes in an MSE High Speed 18.
The pellet was kept in ice and washed in 500ml phosphate buffer pH 7.0. The suspension was re-centrifuged, and the pellet suspended in 33ml ice-cold sucrose (25% sucrose in 0.05M Tris, pH 8.0). 15ml fresh lysozyme (5mg ml⁻¹) in 0.25M Tris, pH 8.0) was added, the suspension shaken gently to mix the contents, then left for 5 minutes at 0°C. 13.5ml cold EDTA (0.25M, pH 8.0) was added and the mixture left in ice for another 10 minutes with occasional swirling. 54ml lysis mixture (1% Brij 58, 0.4% sodium deoxycholate, 0.0625M EDTA in 0.05M Tris, pH 8.0) was added and gently mixed. The suspension was left for 10 minutes or until clear. The lysate was transferred into 50ml polypropylene MSE tubes, centrifuged at 18,000 r.p.m. for 35 minutes at 4°C in an MSE High Speed 18. The supernatant was carefully poured into a measuring cylinder.

(b) Concentration of plasmid DNA

3.2g sodium chloride was added to 110ml cleared lysate and dissolved. 11g polyethylene glycol (PEG) 6,000 was mixed in gently. The lysate was left overnight to allow precipitation of the DNA, centrifuged at 3,000 r.p.m. for 5 minutes in an MSE High Speed 18 and the supernatant poured off. PEG was precipitated by adding sodium chloride to a concentration of 7M. The aqueous layer was poured off after centrifugation and dialysed overnight against 11 TES buffer. The DNA concentration was measured at 260nm on a UV spectrophotometer (Unicam spectrophotometer SP5-500, Pye Unicam, Cambridge) an extinction of 26 being equivalent to a DNA concentration of 1mg ml⁻¹.

(c) Preparation of a competent culture, and transformation

Two different methods were used for the preparation of a competent culture, the first being based on that of Duncan and Tierney (1973). A portion of an overnight culture was diluted one tenth into fresh TY broth and the cells harvested after 3 hours incubation at 30°C. These were washed and resuspended in
saline-EDTA (0.1M sodium chloride and 0.1M EDTA) and DNA added to a concentration of 25 μg ml$^{-1}$. The mixture was incubated for 5 hours before plating on appropriate media.

The second method used was based on that of Moseley and Mattingly (1971) and Tirgari (1977). An overnight culture of *R. trifolii* was diluted into fresh broth and incubated until a log-phase culture of about $3 \times 10^8$ v.u. ml$^{-1}$ was obtained. 10ml was centrifuged and the cells resuspended in 5ml fresh pre-warmed broth and 2ml 0.1M CaCl$_2$. To 0.3ml of this mixture was added 0.05ml transforming DNA and the suspension shaken gently at 30°C for 2 hours. Samples of 0.1ml were then diluted into 10ml fresh medium and incubated overnight, to allow expression of the plasmid genes, before plating on appropriate selective media.

**Transformation: Method 2**

This method of transformation was based on that of Page and Sadoff (1976).

(a) **Preparation of DNA**

A litre of culture was grown to stationary phase. The cells were spun down by centrifugation at 10,000 r.p.m. 4°C on an MSE High Speed 18, washed in half the volume of SSC and finally resuspended in 50ml SSC. To lyse the cells sodium dodecyl sulphate was added to a final concentration of 0.05% and the suspension heated at 60°C for 1 hour with occasional gentle shaking. The crude DNA was used directly for transformation.

(b) **Transformation**

This was carried out on plates of SY medium. A 50μl sample of a log-phase culture containing $2 \times 10^7$ v.u. ml$^{-1}$ was mixed with approximately 3 μg of crude DNA over an area of about 2cm$^2$. After 24 hours incubation at 30°C, the resulting area of growth was scraped off and plated on appropriate selective media.

All transformation experiments included recipient and DNA lysate controls.
An alkaline sucrose gradient analysis of DNA from irradiated and unirradiated populations of *R. trifolii* P3 and *R. trifolii* DC5t to determine whether the latter lacks a correctional endonuclease.

Cells were labelled by growing to a density of approximately $2 \times 10^8$ v.u. ml$^{-1}$ after about 7 generations in 1ml TY broth containing 20 µCi $[^{3}H]3$H thymidine. These were spun down in a bench centrifuge (Quickfit, Corning Ltd., Stone, Staffs.) and resuspended in 1.5ml phosphate buffer. Control samples (0.4ml) were removed and placed on ice. The rest was irradiated with a UV dose of 225J m$^{-2}$, centrifuged, resuspended in pre-warmed TY broth and incubated for 5 minutes at 30°C. Treated and untreated cells were spun down, resuspended in phosphate/EDTA buffer containing freshly dissolved lysozyme at a concentration of 2mg ml$^{-1}$, and left on ice for 10 minutes. 0.1ml samples were layered onto the preformed gradients. These were made from starting solutions of 5, 20 and 40% sucrose dissolved in a solution containing 40.9g l$^{-1}$ NaCl, 12.0g l$^{-1}$ NaOH and 0.3g l$^{-1}$ EDTA. 4.2ml gradients 5-20% (w/v) were formed on top of a 0.2ml shelf of 40% sucrose in MSE polypropylene, and 0.2ml 0.5M NaOH was layered on top of each gradient.

The gradients with the samples were left in the dark for 20 minutes to allow lysis of the cells. They were then centrifuged at 30,000 r.p.m., 20°C for 105 minutes on an MSE Superspeed 65 Ultracentrifuge.

The tubes were removed, pierced and 9 drop fractions collected on strips of filter paper which were allowed to dry overnight. The strips were washed twice in 5% trichloroacetic acid (20 minutes each wash) and twice in absolute ethanol (10 minutes each wash). After drying, the strips were cut up, inserted into scintillation vials to which 10ml toluene-based scintillation fluid (NE 233, Nuclear Enterprises Ltd., Edinburgh) was added. These were counted for 5 minutes each on a Packard Tri-Carb.
liquid scintillation spectrometer, model 3330 (Packard Instruments Ltd., Caversham, Berks.).

Results

Caffeine treatment of UV-irradiated *R. trifolii* P3

*R. trifolii* P3 was treated with a range of concentrations of caffeine (0-200 \( \mu \text{g ml}^{-1} \)) in the post-irradiation medium. The induced mutation frequencies to rifampicin-resistance in each treatment were compared with the induced mutation frequencies in an irradiated but otherwise untreated control. The results are shown in Figure 5-1. Caffeine decreased rather than increased the induced mutation frequency.

Mitomycin C sensitivity of *R. trifolii* P3

As a preliminary to an attempt to isolate an MTC-sensitive mutant of *R. trifolii* P3, which hopefully would also be altered in its susceptibility to mutagenesis (cf. Moseley and Copland, 1978), it was necessary to determine first the lethal effects of this chemical on the wild-type strain. Figure 5-2 shows the results of this experiment, from which a \( D_{37} \) value of 0.03 \( \mu \text{g ml}^{-1} \) hr can be derived. Assuming that the chromosome of *R. trifolii* is similar in size to that of *E. coli*, and using the equation calculated by Moseley and Copland (1975) that a \( D_{37} \) value of 0.038 \( \mu \text{g ml}^{-1} \) hr is equivalent to the production of one cross-link per genome, a single MTC cross-link appears to be capable of completely inactivating *R. trifolii* P3. It was therefore impossible to find a strain which was more sensitive to this agent.

Isolation and properties of a UV-sensitive mutant of *R. trifolii* P3

An experiment was carried out with the intention of isolating a UV-sensitive mutant of *R. trifolii* in the hope that like some UV-sensitive strains of *E. coli* it would be more responsive to the mutagenic effects of UV. The principle of this experiment was
Figure 5-1. The effect of treatment of UV-irradiated \textit{R. trifolii} P3 with caffeine on induced mutation frequency to rifampicin-resistance following exposure to a UV dose of 45J m$^{-2}$.
Figure 5-2. The effect of MTC (1 µg ml⁻¹) on survival of *R. trifolii* P3
based on the finding that UV-sensitive strains of *E. coli* have a considerably reduced ability to repair UV-damage in irradiated phage for which they are host (Ellison, Feiner and Hill, 1960; Witkin, 1967). Such strains are phenotypically host-cell reactivation minus (Hcr⁻). A mutagenised population of bacteria was infected with irradiated phage at a high m.o.i., such that only a few bacteria remain uninfected. Phenotypically Hcr⁺ bacteria were able to reactivate damaged phage and thus were killed by them. Hcr⁻ strains were not able to repair substantial amounts of UV damage, did not reactivate the phage, and thus survived. Only a limited number of bacteria may be spread per plate since at high densities active phage released by Hcr⁺ strains might lyse otherwise unaffected Hcr⁻ strains. The optimum number of bacteria per plate, i.e., 1,000, was determined previously (Howard-Flanders and Theriot, 1962).

A total of 301 survivors from treatment of bacteria with irradiated phage were examined for their UV-sensitivity. One of these was found to be a UV-sensitive mutant and was designated DC5t. The remainder presumably had either escaped phage infection or were phage-resistant. One isolate which was originally thought to be slightly UV-sensitive had a reduced ability to support the growth of phage (10⁻⁵ relative to *R. trifolii* P3).

The extreme sensitivity of *R. trifolii* DC5t to UV can be seen in Figure 5-3. Calibration of the dose rate was from a knowledge of the D₃⁷ value for the wild type. The D₃⁷ values for *R. trifolii* P3 and DC5t were 18J m⁻² and 0.24Jm⁻² respectively, i.e., DC5t was 75 times more sensitive to the lethal effects of UV than *R. trifolii* P3. To confirm that the mutant isolated was indeed a product of the semi-selective method used and not a chance spontaneous mutant, irradiated phage were plated on *R. trifolii* P3 and *R. trifolii* DC5t. If the principle of the isolation method were correct then *R. trifolii* DC5t should lower the efficiency of
Figure 5-3. The effect of UV irradiation on survival of R. trifolii P3 (●) and R. trifolii DC5t (▲)
plating of the irradiated but not unirradiated phage relative to the wild-type due to its inability to repair at least some of the UV damage in the phage DNA. The unirradiated phage was found to plaque equally well on *R. trifolii* DC5t and P3 but, as Figure 5-4 shows, irradiated phage had a reduced efficiency of plating on the former as compared to the latter strain.

**Sensitivity of *R. trifolii* DC5t to MMS and gamma-rays**

If the function which has been mutated in *R. trifolii* DC5t is fairly specific for the repair of UV damage, then the sensitivity to other mutagenic agents such as MMS and gamma-rays should not be altered. Figures 5-5 and 5-6 show the lethal effects of these two mutagenic agents in *R. trifolii* P3 and *R. trifolii* DC5t. In both cases the shape of the survival curve was marginally altered relative to that of the wild-type, in that a slight shoulder was present and the slope was somewhat steeper, i.e. DC5t was fractionally but not significantly more sensitive to the lethal effects of these mutagens in the long term than the wild-type.

The $D_{37}$ values for the wild-type treated with MMS or gamma-rays were 0.05% hr and 2.1 krad respectively and for DC5t 0.06% hr and 2.6 krad respectively.

**Endonuclease incision in *R. trifolii* P3 and DC5t following UV irradiation**

Mutants isolated by Howard-Flanders and Theriot (1962) were subsequently found to map in three loci designated *uvrA*, *uvrB* and *uvrC* (Howard-Flanders, Boyce and Theriot, 1966). These mutants were unable to excise pyrimidine dimers from their DNA (Howard-Flanders and Boyce, 1966), the first two lacking an endonuclease specific for UV-irradiated DNA, while the third had wild-type levels of this enzyme (Braun and Grossman, 1974). Since the function missing in *R. trifolii* DC5t was found to be fairly specific for UV-damage, an experiment was carried out to determine whether this strain might be mutant in a gene analogous
Figure 5-4. Plaqueing of irradiated phage Rtl on *R. trifolii* P3 (●)
*R. trifolii* DC5t (▲)
Figure 5-5. The effect of MMS (0.25%) on survival of *R. trifolii* P3 (●) and *R. trifolii* DC5t (▲)
Figure 5-6. The effect of gamma rays on the survival of *R. trifolii* P3 (●) and *R. trifolii* DC5t (○)
to either uvrA or B in E. coli, i.e. lacking an endonuclease. DNA from irradiated and unirradiated bacteria of the wild-type and UV-sensitive mutant was examined by alkaline sucrose gradient centrifugation.

The results obtained in this experiment were not clear-cut. A small shift in the molecular weight of irradiated DNA from the wild-type strain compared to the unirradiated was noted. An apparently smaller shift in the molecular weight of irradiated DC5t relative to its unirradiated control was also observed, but the resolution of the experiment was such no definite conclusions could be drawn from this result. Further work on the experimental conditions required to show clearly the activity of a correctional endonuclease acting on irradiated DNA of the wild-type was considered necessary before repeating this experiment.

UV mutagenesis in R. trifolii DC5t

The results of a UV mutagenesis experiment on R. trifolii P3 and R. trifolii DC5t are given in Figure 5-7. In this experiment, calibration of the UV dose was against the survival curve of DC5t whose D37 was calculated previously. Induced mutations in DC5t were observed at extremely low doses of UV where virtually no induction of mutation in the wild-type had occurred. The increase over the spontaneous mutation frequency was only about 5-fold. The spontaneous mutation frequency, however, was higher than in the wild-type so that the maximum absolute frequencies induced for P3 and DC5t were 8 \times 10^{-7} (see Chapter 3) and 1 \times 10^{-6} respectively. Thus, induced mutation by UV was only slightly higher in DC5t than the wild-type. The increase in the spontaneous mutation frequency was confirmed for rifampicin-resistance and two other markers, streptomycin-resistance and kanamycin-resistance (see Table 5-1).
Figure 5-7. The effect of UV irradiation on survival (closed symbols) and mutation (open symbols) in *R. trifolii* P3 (● ○) and *R. trifolii* DC5t (▲ △)
Table 5-1. Spontaneous mutation frequencies of *R. trifolii* P3 and *R. trifolii* DC5t

<table>
<thead>
<tr>
<th>Strain</th>
<th>Streptomycin</th>
<th>Kanamycin</th>
<th>Rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>$6.3 \times 10^{-8}$</td>
<td>$2.6 \times 10^{-8}$</td>
<td>$3.0 \times 10^{-8}$</td>
</tr>
<tr>
<td>DC5t</td>
<td>$1.2 \times 10^{-7}$</td>
<td>$1.6 \times 10^{-7}$</td>
<td>$1.2 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Table 5-2. Analysis of the cross *R. trifolii* uvs-1 rif-2 (R68.45) x *R. leguminosarum* ser-2 ade-88 str-1

<table>
<thead>
<tr>
<th>Selected donor marker</th>
<th>Non-selected markers of recombinants</th>
<th>No. of R$^+$ recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ade$^+$</td>
<td>uvs$^+$ ser</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>uvs$^+$ ser</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>uvs$^+$ ser$^+$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>uvs$^+$ ser$^+$</td>
<td>1</td>
</tr>
<tr>
<td>ser$^+$</td>
<td>uvs$^+$ ade</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>uvs$^+$ ade$^+$</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>uvs$^+$ ade$^+$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>uvs$^+$ ade$^+$</td>
<td>1</td>
</tr>
</tbody>
</table>

Linkage: ade - ser = 3%
          ade - uvs = 24%
          ser - ade = 2%
          ser - uvs = 18%
Preliminary experiments were carried out to determine whether plasmid R68.45 was capable of conjugational transfer of markers from *R. trifolii* to *R. leguminosarum*. A rifampicin- and streptomycin-resistant derivative of *R. trifolii* was isolated and the plasmid R68.45 transferred into this strain from an *E. coli* donor to give *R. trifolii* DC4t. A cross was carried out with a spectinomycin-resistant derivative of *R. leguminosarum* 300 (strain DC31) as recipient. Selection was made both for separate and simultaneous transfer of the two markers, since in *R. leguminosarum* rif and str are closely linked (Beringer, Hoggan and Johnston, 1978). The rif marker transferred at a frequency $1.1 \times 10^{-6}$ per recipient, str at $1.5 \times 10^{-6}$ per recipient and rif str at $1.5 \times 10^{-6}$ per recipient. Thus, R68.45 was capable of transfer of markers from *R. trifolii* to *R. leguminosarum* at frequencies similar to those of R68.45-mediated marker transfer between strains of *R. leguminosarum* and from *R. leguminosarum* to *R. trifolii* (Johnston and Beringer, 1977; Beringer, Hoggan and Johnston, 1978). The simultaneous transfer of the rif and str alleles at almost the same frequency as their individual transfer strongly suggests that these two markers are also closely linked in *R. trifolii*, although this was not confirmed by analysis of the co-inheritance frequencies of the non-selected markers where single marker transfer was selected.

Having confirmed R68.45-mediated marker transfer in *R. trifolii*, a suitable donor strain of *R. trifolii* DC5t was constructed by isolation of a rifampicin-resistant mutant and transfer into this strain of R68.45 from an *E. coli* donor (i.e. DC8t). In preliminary experiments the uvs marker was found to map in Section 7 of the chromosome of *R. leguminosarum* (see Figure 2-2). Further crosses were carried out with
R. leguminosarum DC11 as recipient, to ascertain the position of this marker within the section. Selection was either for \( \text{ser}^+ \) or \( \text{ade}^+ \) these being the two flanking markers for this section.

Exconjugants were then tested for UV-sensitivity by streaking them across a plate and irradiating one half of this with a UV dose of 22.5 J m\(^{-2}\) for resistance to rifampicin, kanamycin and tetracycline and for prototrophy, i.e. transfer of the second flanking marker. Clones showing resistance to rifampicin, i.e. spontaneous streptomycin-resistant mutants of \( R. \) trifolii, or sensitivity to kanamycin and tetracycline, i.e. spontaneous prototrophic revertants of \( R. \) leguminosarum or \( R^- \) recombinants, were excluded from the analysis (see Table 5-2).

The uvs marker was located approximately in the middle of Section 7, being 24% linked to \( \text{ade}^{-88} \) and 18% linked to \( \text{ser}^{-2} \) (see Figure 5-8).

Attempts to transfer into \( R. \) trifolii plasmids known to enhance UV-mutagenesis

(a) Direct conjugational transfer

Conjugational transfer to \( R. \) trifolii of five plasmids known to enhance UV-resistance and mutagenesis (see Table 2-4) was attempted. In conjugational experiments there was no evidence of transfer of pKM101 from either TA100 or E. coli 24; of pMG1, R931 or R2 from P. aeruginosa PU21; or of R751-SU2[ from E. coli W677 or P. putida AC34 to appropriate recipient strains derived from \( R. \) trifolii DC5t. Attempts to transfer pKM101 from S. typhimurium TA100 to two other strains of \( R. \) trifolii, DC01t and DC001t, were similarly unsuccessful.
Figure 5-8. Position of the uvs marker on the chromosome of *R. leguminosarum*. Numbers above the arrowed lines are co-inheritance percentages for the alleles shown.
(b) Direct conjugation of plasmids following inhibition of the restriction and modification system of the recipient rhizobia

The possibility was considered that restriction and modification of incoming plasmid DNA might be lowering the number of rhizobia carrying the plasmid to a frequency which was not detectable. The restriction ability of *Rhizobium* can be reduced by a short heat treatment at temperatures around 47°C (Schwinghamer, 1966; Boucher et al., 1977). A 7 minute heat treatment of the recipient *R. trifolii* DC7t immediately prior to mating reduced the viability of the population by 50% and increased approximately 6-fold the frequency of transfer of the plasmid R702, from $6 \times 10^{-2}$ per recipient to $3.5 \times 10^{-1}$ per recipient for the unheated and heat-treated bacteria respectively. This method was then used in the crosses *E. coli* 24-3 (carrying pKM101) × *R. trifolii* DC6t, *P. aeruginosa* PU21 carrying PMG1, R931 or R2 × *R. trifolii* DC7t, and *E. coli* W677 (R751-SU2) × *R. trifolii* DC7t but there was still no evidence of transfer of any of these plasmids.

(c) Attempts to mobilise pKM101 a plasmid transferable to *R. trifolii*

The plasmid R702 was chosen to attempt the mobilisation of pKM101 since it does not code for ampicillin-resistance and transfers to *R. trifolii* at high frequencies (see above). A strain of *E. coli* J5-3 carrying both these plasmids was constructed by the transfer of pKM101 from *E. coli* 24-2 to *E. coli* J5-3 (R702). Crosses were first carried out using *E. coli* 24-1 as a recipient for this donor strain, in order to investigate transfer of either or both of these plasmids (see Table 5-3). The presence of the plasmid pKM101 appeared to inhibit transfer of the plasmid R702 almost 10-fold whereas pKM101 was transferred at a high frequency. In all cases where transfer of R702 was selected, the plasmid pKM101 was present, whereas selection for pKM101
<table>
<thead>
<tr>
<th>Cross</th>
<th>Plasmid selected</th>
<th>Transfer frequency per recipient</th>
<th>Proportion of exconjugants with non-selected plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli J5-3(R702) x E. coli 24-1</td>
<td>R702</td>
<td>$2 \times 10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td>E. coli J5-3(R702)(pKM101) x E. coli 24-1</td>
<td>R702</td>
<td>$3 \times 10^{-4}$</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>pKM101</td>
<td>$5 \times 10^{-2}$</td>
<td>0/100</td>
</tr>
<tr>
<td></td>
<td>R702 and pKM101</td>
<td>$2 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>E. coli J5-3(R702)(pKM101) x R. trifolii DC6t</td>
<td>R702</td>
<td>$3 \times 10^{-5}$</td>
<td>0/100</td>
</tr>
<tr>
<td></td>
<td>pKM101</td>
<td>$&lt; 5 \times 10^{-8}$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R702 and pKM101</td>
<td>$&lt; 5 \times 10^{-8}$</td>
<td>-</td>
</tr>
</tbody>
</table>
yielded less than 1% also carrying R702. This suggests that transfer of R702 is being controlled in some manner such that transfer of R702 from this donor requires transfer of pKM101, but transfer of the latter may also proceed independently of R702. On the basis of these results simultaneous selection for transfer of R702 and pKM101 would be expected to show a frequency of transfer identical to that for R702 alone, but this frequency is in fact about one-tenth lower. The reason for this difference is not clear but may at least partly reflect lower efficiencies of plating of the bacteria on media containing two antibiotics.

Although it appeared that pKM101 might be regulating transfer of R702 in some manner, a cross was carried out to investigate transfer of these two plasmids to R. trifolii DC1t from an E. coli strain carrying them both. As previously, selection was imposed for individual and simultaneous transfer of these plasmids. The transfer of R702 from this donor strain to R. trifolii was also inhibited, the frequency being $3 \times 10^{-5}$ compared to $6 \times 10^{-2}$ for a previous observation (see above). A total of 213 R702-containing exconjugants were screened for ampicillin-resistance, i.e. pKM101 transfer, but none were found. No transfer of pKM101 was observed when selected for independently, or simultaneously with R702.

(d) Attempts to obtain recombinant plasmids, with UV-enhancing properties, which are transferable to R. trifolii

P2 and P9 group plasmids are not transmissible to E. coli, so that selection for transfer of plasmids belonging to either of these groups to recipients of this species from a P. aeruginosa donor also containing a P1 plasmid allowed the isolation of plasmids which were found to be recombinants (Hedges and Jacob, 1975; Jacoby, Jacobs and Hedges, 1976). The P2 and P9 hybrids used in this experiment were all capable of enhancing UV-resistance and mutagenesis. The P1-P2 hybrids had lost this
property but the plasmid R751-SU2, a P1-P9 hybrid had retained it, at least in P. putida (G.A. Jacoby, personal communication).

A similar method was used in an attempt to obtain hybrid plasmids of RP4 and R931, and RP4 and R2 (RSU2). Donor strains of P. aeruginosa PU2 carrying RP4 and R931, or RP4 and R2, were constructed. Crosses were carried out with E. coli CSH29 as recipient and selection was made for the transfer of streptomycin-resistance, i.e. R931 or R2 markers. Although RP4 was found to transfer at a frequency of \( 3 \times 10^{-4} \), no evidence was found for the transfer of either R931 or R2 to E. coli.

(e) Attempts to transform R. trifolii DC2t with the plasmid pKM101

The possibility was considered that transfer of the plasmid pKM101 was not occurring simply due to lack of formation of mating pairs between the plasmid-containing donor and the R. trifolii recipient. It was therefore decided to attempt transformation of R. trifolii by this plasmid by two different methods. The first involved the preparation of donor DNA from E. coli 24-2 by a means designed to increase the proportion of plasmid relative to chromosomal DNA and subsequent transformation by two different procedures. The second involved the preparation of a crude lysate also from E. coli 24-2 and a plate transformation procedure. The recipient in each case was R. trifolii DC2t. DNA was also prepared from R. trifolii DC1t by the latter method and selection was made for transfer of the chromosomal rif marker. In none of these experiments was there evidence of transformation by either the plasmid (ampicillin-resistance) or chromosomal marker (rifampicin-resistance).
Discussion

Neither the caffeine treatment of UV-irradiated *R. trifolii* nor an attempt to isolate a MTC-sensitive mutant of this species in an effort to enhance its susceptibility to mutagenesis was successful. A semi-selective procedure for the isolation of UV-sensitive mutants was, however, successful, one such mutant of *R. trifolii* being isolated and studied. A UV-sensitive mutant of *R. trifolii*

The mutant *R. trifolii* DC5t was 75 times more sensitive than its parental strain to the lethal effects of UV, a figure comparable to the relative sensitivity of a *uvrA* strain of *E. coli* which was 60 times more sensitive than the wild-type (Howard-Flanders and Boyce, 1966). *R. trifolii* DC5t was unable to host-cell reactivate irradiated phage as predicted by the method of isolation and was not significantly more sensitive to the lethal effects of MMS and gamma-rays, suggesting that the unimpaired repair function in this strain was fairly specific for UV damage. The phenotype of this mutant including its response to UV mutagenesis (see below) suggested it lacked excision repair and that it was probably analogous to the *uvrA* or *B* mutants of *E. coli*, i.e. lacked a correctional endonuclease for the repair of UV-induced damage. An experiment to determine whether such an endonuclease function was missing in *R. trifolii* DC5t did not yield unequivocal results. The possibility that this mutant was analogous with the *uvrC* (i.e. lacking a gene product preventing the endonuclease incision from being resealed), or *polA* (i.e. lacking polymerase activity required to fill in gaps produced opposite dimers), mutants of *E. coli* which are also defective in excision repair could not be completely ruled out.

Surprisingly the spontaneous mutation frequency, at least for three markers, was found to be enhanced in *R. trifolii* DC5t suggesting that the missing function must be involved in the
repair of at least some damage introduced spontaneously into the DNA. UV-induced mutation in this strain occurred at very low doses which had little effect on the wild-type parental strain either with respect to its mutagenic or lethal response. This has also been observed in Uvr" strains of E. coli (Witkin, 1966; Bridges and Munson, 1966). It can be calculated from the induced mutation frequencies of the wild-type \(2 \times 10^{-8}\) and UV-sensitive mutant \(8 \times 10^{-7}\) that at a dose of \(0.9 J m^{-2}\) a thymine dimer in a rif gene of DC5t is 40 times more likely to give rise to a mutation than the same in the wild-type strain as a result of being dealt with by pathway(s) other than excision repair.

From a practical point of view, however, the maximum induced mutation frequency for DC5t was only slightly higher than that of the wild-type. One of the factors limiting the maximum number of mutants in the former strain was its extreme sensitivity to the lethal effects of UV. It was therefore decided to attempt transfer into this strain of a plasmid known to enhance UV survival and mutagenesis. Such plasmids probably produce their effects by an enhancement of the error-prone pathway of repair (see Introduction to this Chapter). It was anticipated, therefore, that such a plasmid in a UV-sensitive mutant of R. trifolii P3 would increase mutability by enhancing the probability of mutation per dimer and simultaneously enhancing the number of dimers which can be dealt with by this error-prone pathway, i.e. increasing survival.

Attempts to transfer into R. trifolii plasmids known to enhance UV survival and mutagenesis

Attempts were made to transfer to R. trifolii by conjugation five plasmids known to enhance UV mutagenesis and survival. These included plasmids belonging to incompatibility groups N (pKM101), P2 (pMG1 and R931), P9 (R2) and a P1-P9 hybrid reported as having P1 group properties (R751-SU2; Hedges and
Beringer (1974) was unable to demonstrate transfer to R. leguminosarum of plasmids belonging to incompatibility groups other than P (i.e. P1) and as far as the author is aware there have been no published reports of transfer to Rhizobium of plasmids other than those belonging to incompatibility group P1. It is therefore perhaps not surprising that there was no evidence of transfer of the first four of the plasmids mentioned above.

The plasmid R751-SU2, however, which was reported as belonging to the incompatibility group P1 was not transmissible to R. trifolii. This plasmid was also not transferable to P. aeruginosa (G.A. Jacoby, personal communication), a species which can also act as recipient for plasmids of this incompatibility group. It would therefore appear that, although this plasmid appears to have incompatibility properties of group P1 when in E. coli, these are not functioning with respect to its ability to transfer to other host species.

Lack of transfer of the UV-enhancing plasmids may have been due to restriction and modification of incoming DNA, inability to form mating pairs between donor and recipient strains or inability of the recipient Rhizobium to stably maintain the plasmid. Restriction and modification by the recipient rhizobia was inhibited by heat treatment but there was still no evidence of transfer of any of these plasmids. Attempts to overcome problems associated with formation of mating pairs by transformation of R. trifolii with the plasmid pKM101 were also unsuccessful. It seemed likely that the correct conditions for transformation had not been met since there was no evidence of transformation of this species by a chromosomal rif marker from another strain. An attempt to mobilise this plasmid by R702, which was transferable to R. trifolii at high frequencies, was also unsuccessful. A preliminary experiment to investigate
transfer of these two plasmids from a single donor to an *E. coli* recipient indicated that pKM101 was probably controlling transfer of R702 and not vice versa. If these observations held true for transfer of these two plasmids from a single donor strain to *R. trifolii*, then perhaps one would not expect to detect transfer of pKM101.
Chapter 6

REGULATORY EFFECTS OF AMMONIUM IONS ON THE CLOVER-RHIZOBIUM SYMBIOSIS
Introduction

Combined nitrogen can inhibit nodulation by rhizobia of their host legume (Munns, 1968a; Dart and Wildon, 1969; Hinson, 1975). Nitrate, nitrite, ammonium and urea inhibit various stages of the nodulation process including the number of infections, time of appearance of the first nodule and the number and weight of nodules formed, at concentrations not completely inhibitory to nodule formation (Gibson and Nutman, 1960; Nutman, 1962; Darbyshire, 1966; Munns, 1968a). It has been suggested, at least in the case of nitrate, that this inhibition is due to a destruction of the plant hormone indole-acetic acid which is thought to be important in the nodulation process (Tanner and Anderson, 1963; Munns, 1968b).

Combined nitrogen can also inhibit nitrogen fixation by a nodulated legume (Allos and Bartholomew, 1959; Hinson, 1975; Kamberger, 1977). Inhibition was observed with inorganic sources of nitrogen such as ammonium, nitrate or nitrite but not organic sources such as glutamine, glutamic acid or aspartic acid even though these were capable of supporting plant growth (Kamberger, 1977). The manner in which combined nitrogen inhibits symbiotic nitrogen fixation has not been clarified.

Houwaard (1977) observed inhibition by ammonium of nitrogenase activity of intact pea plants and detached nodules, but no decrease in the nitrogenase activity of bacteroids isolated from these. He suggested that inhibition was due to a reduced supply of photosynthate to the bacteroids. Bisseling, van den Bos and van Kammen (1978) found no evidence of an inhibition by ammonium nitrate of nitrogenase synthesis within bacteroids although the nitrogen-fixing activity of intact nodules did decrease. They correlated this latter observation with a decrease in the amount of leghaemoglobin within the nodule. A third explanation compatible with the effect of nitrogen on nitrogen fixation in
free-living bacteria is that ammonium, possibly acting via glutamine synthetase of plant origin, is responsible for inhibition of nitrogenase activity (Kamberger, 1977), although genetic evidence (see Chapters 1 and 7) suggests that the bacterial glutamine synthetase may be responsible.

It was hoped to isolate mutants of **R. trifolii** which were derepressed for nitrogen fixation (see Chapter 7) i.e. mutants in which nitrogenase activity was not inhibited by ammonium to the same extent as the parental strain. It was therefore necessary to determine the concentration of ammonium required to inhibit nitrogenase activity (acetylene reduction) of nodulated clover plants in the test system being used. The inhibition of nodulation by ammonium was also investigated to ascertain whether the combined nitrogen source for the inhibition of fixation could be applied prior to nodulation of the plants or whether it must be applied at a later stage.

**Materials and Methods**

**Inhibition of nodulation by ammonium ions**

Clover plants were set up as described in Chapter 2 except that the growth medium (liquid and solidified) contained ammonium ions as ammonium sulphate, at concentrations ranging from 0 to 10mM. Treatments were replicated (eight to ten replicates each) and randomised throughout the tube racks being used. Randomisation was carried out by assigning to each position of the racks being used a number, the total positions being assigned corresponding to the number of plants to be tested. The various treatments, including replicates, were written out in an orderly fashion. A table of random numbers was then consulted (Fisher and Yates, 1963) and each successive plant treatment was assigned a number corresponding to a position number in the test-tube racks, and the plants placed accordingly. Nodules
were counted when the plants were 30 days old.

The effect of ammonium ions on the growth of clover plants

Uninoculated clover plants were set up in ammonium-containing growth medium, as above. Treatments were replicated and randomised as described previously. The plants were harvested when 30 days old and dried at 80°C for 20 hours before weighing.

The inhibition of acetylene reduction (nitrogen fixation) by ammonium ions

Five week old plants were used in these experiments. Ammonium salts and their corresponding potassium salts were dissolved in distilled water at appropriate concentrations and the pH was adjusted to a value of 7.0. These solutions were added to plants in quantities sufficient to cover the roots following removal of the liquid plant growth medium. Acetylene reduction assays were carried out as described in Chapter 2. Treatments were replicated, and randomised as described above.

Results

The inhibition of nodulation by ammonium ions

The complete inhibition of nodulation occurred at a concentration of 5mM ammonium (see Figure 6-1), which was not much greater than the minimum amount required to fully support growth of the clover plants, i.e. approximately 1mM ammonium (see Figure 6-2). At a concentration of 0.1mM ammonium an increased number of nodules compared to the untreated control was found. This stimulatory effect of low levels of nitrogen was observed previously (Nutman, 1962; Hinson, 1975) and was attributed partly to a stimulation of growth of the plant combined with a delay in the onset of nodulation such that a greater area of root hair surface was available for the formation of nodules.
Figure 6-1. The effect of ammonium on nodulation of *T. repens* cv. Huia by *R. trifolii* P3. Values are mean of at least 8 replicates ± 1 standard deviation.

Figure 6-2. The effect of ammonium on dry weight of *T. repens* cv. Huia. Values are mean of 5 replicates ± 1 standard deviation.
The inhibition of acetylene reduction (nitrogen fixation) by ammonium ions

A preliminary experiment was carried out with a few ammonium salts, and the corresponding potassium salt as control, to determine which was the most suitable for the investigation of inhibition of acetylene reduction. Since only major differences in the effects of these chemicals were sought, each treatment included only three replicates. The results are shown in Table 6-1. The values obtained for the untreated control were rather variable. Nevertheless, in all cases the ammonium salts tended to inhibit acetylene reduction to a greater extent than the corresponding potassium salt, but in all cases except potassium acetate these also inhibited acetylene reduction. Ammonium acetate was therefore chosen for a further investigation.

The effect of different concentrations of ammonium and potassium acetate on acetylene reduction was investigated, and the results are shown in Figures 6-3 and 6-4. As previously, considerable variability in the nitrogenase activity of different replicates was observed although under the constraint of inhibition by ammonium ions the range of this variability was reduced (see Figure 6-3). Both ammonium and potassium acetate inhibited the reduction of acetylene to ethylene although inhibition by the former was greater at all concentrations tested. In both cases the inhibition increased with concentrations up to approximately 100mM. A further doubling of concentration had little effect. It was not possible to tell from this experiment whether the relatively small amount of ethylene produced by plants treated with 100mM and 200mM ammonium was the product of a period of uninhibited nitrogenase activity followed by a period of complete inhibition or of a period during which nitrogenase activity was considerably, but not completely, reduced. A further experiment was therefore carried out in which plants
The effect of various ammonium and potassium salts (20mM NH\textsubscript{4}\textsuperscript{+} or K\textsuperscript{+}) on the reduction of acetylene to ethylene by *T. repens* cv Huia nodulated by *R. trifolii* DCl1t

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative amounts of ethylene produced per plant in 40 hr (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1.00 ± 1.03</td>
</tr>
<tr>
<td>CH\textsubscript{3}COOK</td>
<td>1.00 ± 0.29</td>
</tr>
<tr>
<td>CH\textsubscript{3}COONH\textsubscript{4}</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>K\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}</td>
<td>0.35 ± 0.29</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4}</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>K\textsubscript{2}SO\textsubscript{4}</td>
<td>0.59 ± 0.68</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</td>
<td>0.20 ± 0.15</td>
</tr>
</tbody>
</table>

(a) Mean of 3 replicates ± 1 standard deviation
Figure 6-3. The effect of ammonium acetate on nitrogenase activity of *T. repens* cv. Huia nodulated by *R. trifolii* DC1t as measured by the acetylene reduction assay value are the mean of at least 8 replicates ± 1 standard deviation.

Figure 6-4. The effect of potassium acetate on nitrogenase activity of *T. repens* cv. Huia nodulated by *R. trifolii* DC1t as measured by the acetylene reduction assay. Values are the mean of at least 8 replicates ± 1 standard deviation.
treated with 100mM ammonium or potassium acetate were assayed for ethylene production at various time intervals.

The results of this experiment are shown in Figure 6-5. As before, potassium acetate inhibited acetylene reduction slightly but the inhibition by ammonium acetate was greater. The inhibition by ammonium ions appeared to occur very rapidly. Even after 9 hours, although the differences were small at this stage, the ammonium-inhibited plants had produced less ethylene than the untreated controls. The production of ethylene proceeded at a constant rate, 0.8 nmoles plant⁻¹ hr⁻¹, after the initial lag which was observed for all treatments, although this rate was considerably reduced compared to that of the untreated control, 15 nmoles plant⁻¹ hr⁻¹. A higher concentration of ammonium would presumably completely inhibit nitrogenase activity, although the previous experiment suggested this would probably be much greater than 100mM.

In practice, the concentrations of ammonium ions surrounding the roots would be slightly less than the concentration applied due to diffusion of ammonium ions into the nitrogen-free plant growth agar. Since the aim of this experiment was to determine the concentration of ammonium ions which must be applied to inhibit acetylene reduction, it was not considered necessary to know the final concentration of ammonium ions surrounding the roots at the time of the assay.

Discussion

Ammonium ions were observed to inhibit both nodulation and acetylene reduction (nitrogenase activity) in clover plants infected with *R. trifolii* DC1t. A concentration of 5mM ammonium ions surrounding the roots of these plants completely inhibited nodulation. It is difficult to compare this figure with previous observations since these were carried out with different symbiotic
Figure 6-5. The effect of 100mM ammonium acetate (▼), 100mM potassium acetate (▼) and distilled water (■) on nitrogenase activity of *T. repens* cv. Huia nodulated by *R. trifolii* DC1t as measured by the acetylene reduction assay of various time intervals. Values are the mean of at least 8 replicates ± 1 standard deviation.
associations and different test systems.

The exact concentrations of ammonium ions surrounding the roots required for inhibition of acetylene reduction was not determined, but applied concentrations between 10mM and 100mM were found to have increasing inhibitory effects on acetylene reduction. Kamberger (1977), using a test tube system for growth of alfalfa plants, observed an inhibition of nitrogenase activity with concentrations of ammonium sulphate between 10 and 100mM. At the latter concentration nitrogenase activity was completely inhibited. Houwaard (1977) observed a 20 to 40% drop in acetylene reducing activity of pea plants treated with 20mM ammonium chloride. Therefore, inhibition of nitrogenase activity by ammonium in these three symbiotic associations occurs at similar concentrations.

The inhibition of nodulation by ammonium ions occurred at a concentration slightly higher than was required for the growth of the plant. This implies a form of regulatory mechanism whereby the establishment of symbiosis occurs only under conditions where this is beneficial for the host plant. Nodulation of the plants by rhizobia where the former had a sufficient supply of nitrogen would, in effect, be a parasitic association. A 100mM concentration of ammonium ions applied to the plants was required for an almost maximal decrease in nitrogenase activity. This demonstrates that a much higher concentration of ammonium was required for the inhibition of nitrogenase activity than for the inhibition of nodulation. Bacteroids fixing nitrogen could presumably rapidly accumulate relatively high concentrations of ammonium ions within a nodule even though the rest of the plant might require nitrogen. It would therefore be inefficient to "shut down" nitrogenase activity at relatively low concentrations of ammonium ions since these would be translocated eventually from the nodule and this activity would need to be resumed.
symbiotic association has presumably evolved a regulatory response to nodule concentrations of ammonium ions which are relevant to the plant as a whole.
Chapter 7

SYMBIOTIC MUTANTS OF RHIZOBIUM TRIFOLII
Introduction

In Chapter 1 it was pointed out that symbiotic mutants of Rhizobium can be isolated directly or indirectly. Using the direct approach Meade et al. (1979) obtained symbiotically defective mutants of R. meliloti. The genes involved were located on the chromosome although recent evidence has suggested that at least some of the genes involved in the Rhizobium-legume symbiosis may be plasmid-located (Dunican and Cannon, 1970; Dunican, O'Gara and Tierney, 1976; Johnston et al., 1978; Prakash et al., 1978 quoted in Hirsch, 1979).

Genetic evidence has suggested that the enzyme glutamine synthetase (GS) may be involved in the regulation of nitrogen fixation in Rhizobium, as it is in the free-living nitrogen-fixing bacterium Klebsiella pneumoniae (see Chapter 1). Thus, an indirect approach involving the isolation of mutants having an altered GS may give strains which also have altered symbiotic properties. Mutants having an altered GS may be isolated in a number of different ways. Glutamine-requiring auxotrophs of Rhizobium cowpea (Ludwig and Signer, 1977) and K. pneumoniae (Tubb, 1974; Streicher et al., 1974) obtained following screening procedures were unable to fix nitrogen. Some glutamine-requiring auxotrophs of K. pneumoniae which also lacked the enzyme glutamate synthase (GOGAT) were derepressed for nitrogen fixation (Shanmugam, Chan and Morandi, 1975), as were other mutant strains of this species having constitutive levels of GS (Streicher et al., 1974). Of six prototrophic revertants of Rhizobium cowpea, four were found to have regained their catalytic but not regulatory properties, thereby demonstrating that these two activities of the enzyme can be separated (Ludwig and Signer, 1977). Glutamine-requiring auxotrophs of R. meliloti which were ineffective have been isolated following a selective procedure designed to detect D-histidine-utilising
derivatives of a his mutant of this species (Kondorosi et al., 1977b). This experiment was based on the observation of Kustu and McKereghan (1975) that D-histidine-utilising revertants of a his- mutant of Salmonella typhimurium either had a low level of GS (<10% of parental activity) or a high level of the inactive form of this enzyme. They suggested that GS might be involved in the regulation of the D-histidine racemising enzyme such that the activity of the latter was increased when the GS level was low or this enzyme was in its inactive form.

Both GS and GOGAT are inhibited by the glutamate analogues methionine sulfoximine (MSX) and methionine sulphone (MS) although the latter inhibits GS more than it inhibits GOGAT (Brenchley, 1973). A mutant of R. trifolii resistant to MSX had a number of defects in carbon and nitrogen metabolism and was able to fix nitrogen in the free-living form (O'Gara and Shanmugam, 1977). Levels of GOGAT and glutamate dehydrogenase in this mutant were lowered but GS was unaffected.

This chapter describes the isolation of some symbiotic mutants of R. trifolii. Firstly, a direct approach involving transposon mutagenesis and screening of clones was used. Secondly, attempts were made to isolate glutamine auxotrophs in the hope that these might have altered symbiotic properties. The third approach involved the isolation of MS- and MSX-resistant mutants, again in anticipation of these having altered symbiotic properties and in particular that they might be less sensitive to the inhibitory effects of ammonium ions, i.e. derepressed.

Materials and Methods

The direct isolation of symbiotic mutants

A cross between E. coli 1830 and R. trifolii DC1t was set up. Following incubation, the bacteria from this cross were suspended
in TY broth and plated onto an appropriate selective medium to
detect the transfer of Tn5 to _R. trifolii_ DC1t. The frequency of
transfer was $10^{-6}$ and a total of approximately 1,000 colonies
was obtained following incubation. Individual colonies were
transferred to 1ml TY broth in test tubes and incubated for 2 days
at 30°C. The cultures were diluted 100-fold into liquid Jensen's
medium and each used as an inoculum for an individual plant. A
0.5ml volume from each culture was also diluted one half into
40% glycerol and these stocks were stored at -20°C.

The plants were grown until 5 weeks old and acetylene reduc-
tion assays were carried out as described in Chapter 2. Puta-
tive non-nodulating and ineffective mutants were re-tested, five
replicates being used for each test.

Two mutants selected for further study were tested at two
different temperatures, 15°C and 25°C. Plants at 15°C were
grown in a growth room at 15°C ± 1°C illuminated by Wotam mercury
iodide lights delivering 63,000 lux at plant height. Plants at 25°C
were grown in a greenhouse with a minimum temperature of
25°C, and lighting as described previously (see Chapter 2).
Treatments were replicated, and randomised as described in
Chapter 6. The nodulated plants were assayed for acetylene
reduction at the appropriate temperature when 5 weeks old. Dry
weight measurements were also made.

**Isolation and testing of bacteria from nodules**

Nodules were sterilised by soaking in 95% ethanol (1 minute),
followed by 0.1% mercuric chloride (3 minutes), then washing
six times in sterile distilled water. Each individual nodule
was crushed in 1ml TY broth and dilutions were spread on TY
plates. After 3 days incubation isolated colonies were picked
and tested for kanamycin resistance and rifampicin resistance.

**Mapping of the site of the transposon insertion giving rise to the
Eff-2 phenotype**

An appropriate donor strain was constructed by the transfer of
the plasmid pJB3J1 from _E. coli_ 1843 to _R. trifolii_ DC21t. This was used in crosses with appropriate recipient strains of _R. leguminosarum_ (see Table 2-2). Screening was for kanamycin resistance (Tn5).

The indirect isolation of symbiotic mutants

(a) Attempts to isolate glutamine-requiring auxotrophs

Two experiments for the isolation of auxotrophs have been described previously (see Chapter 3). The minimal medium used in these experiments contained 0.5mM ammonium chloride as nitrogen source instead of glutamate so that it would be possible to detect glutamine auxotrophs if such were present. In a third experiment an MNNG-mutagenised culture was washed twice and diluted into minimal medium, again with 0.5mM ammonium chloride as the nitrogen source, to give approximately 10^7 v.u. ml⁻¹. This culture was allowed to grow for 4 hours before addition of ampicillin to a concentration of 1mg ml⁻¹. A preliminary experiment had shown that exposure to this concentration of antibiotic for 4 hours at 30°C reduced the viable numbers in a culture of _R. trifolii_ P3 to 10%, but barely affected a culture of a _met^- mutant of _R. trifolii_. Following a similar treatment, the mutagenised culture was plated for the detection of auxotrophs as described in Chapter 2. Putative auxotrophs were tested for their ability to grow on SY minimal medium with ammonium chloride, glutamate or glutamine as nitrogen source.

An indirect approach to the isolation of glutamine-requiring auxotrophs involved an attempt to obtain D-histidine utilising mutants of _R. trifolii_ DC19t, using a gradient plate technique based on that of Schwinghamer (1968). 10ml amounts of SY medium were poured into plates and allowed to set at a slight angle. A top layer of medium with half-strength agar containing 3mM D-histidine, 15g l⁻¹ sucrose, and 0.1ml of the culture, was poured onto the base layer. The plates were incubated for 5 days at 30°C.
(b) The isolation of mutants resistant to methionine sulphone or methionine sulphoximine

Two different methods were used. In the first method, methionine sulphoximine (MSX; 10mM) or methionine sulphone (MS; 7.5mM) were incorporated into plates of SY medium having as nitrogen source 0.5mM ammonium chloride. Unirradiated and UV-irradiated (67.5J m\(^{-2}\)) cultures were spread on these plates which were examined after 5 days incubation at 30°C.

The second method employed was the gradient plate technique except that 0.5mM ammonium chloride was the nitrogen source and MSX (15mM) or MS (10mM) replaced D-histidine. After 5 days incubation putative resistant colonies were restreaked on minimal medium containing MSX (10mM) or MS (7.5mM).

Strains confirmed as being resistant to either of these analogues were tested on plants. MSX-resistant strains were tested for derepression of acetylene reduction (nitrogen fixation) in the presence of 100mM acetate, this experiment being carried out as described in Chapter 6.

Results

Isolation of symbiotic mutants: a direct approach

Approximately 800 clones from a transposon mutagenesis experiment were divided into three batches and tested on plants one batch at a time. A total of six putative ineffective mutants were isolated. Two of these, *R. trifolii* DC20t and DC21t, which were obtained in the first batch, were studied further. Only preliminary data (see Table 7-1) were obtained for four others which were isolated later.

*R. trifolii* DC20t and DC21t were tested at two different temperatures, 15°C and 25°C. Acetylene reduction and dry weight measurements of nodulated plants were made and rhizobia
Table 7-1. Acetylene reduction by four putative ineffective mutants of *R. trifolii* nodulating *T. repens* cv. Huia

<table>
<thead>
<tr>
<th>Inoculum strain</th>
<th>Acetylene reduction; nmoles ethylene formed per plant in 40 hr (a)</th>
<th>Acetylene reduction; relative formation of ethylene (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC1t</td>
<td>$1.6 \times 10^3 \pm 0.6 \times 10^3$</td>
<td>$1.0 \pm 4.1 \times 10^{-1}$</td>
</tr>
<tr>
<td>DC23t</td>
<td>$4.4 \times 10^0 \pm 10.2 \times 10^0$</td>
<td>$2.8 \times 10^{-3} \pm 6.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>DC24t</td>
<td>$9.6 \times 10^1 \pm 7.7 \times 10^1$</td>
<td>$6.1 \times 10^{-2} \pm 4.9 \times 10^{-2}$</td>
</tr>
<tr>
<td>DC25t</td>
<td>$5.8 \times 10^1 \pm 6.9 \times 10^1$</td>
<td>$3.7 \times 10^{-2} \pm 4.4 \times 10^{-2}$</td>
</tr>
<tr>
<td>DC26t</td>
<td>$4.9 \times 10^1 \pm 4.5 \times 10^1$</td>
<td>$3.1 \times 10^{-2} \pm 2.8 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

(a) Mean of 5 replicates $\pm 1$ standard deviation
isolated from nodules were tested to find if they still carried Tn5 (Km) and the results are given in Tables 7-2 and 7-3. These show that strains DC20t and DC21t are at least partly ineffective relative to the parental strain.

*R. trifolii* DC21t, in particular, had considerably decreased acetylene reducing ability, i.e. nitrogenase activity relative to the parental strain at both 15°C and 25°C. Dry weights of plants nodulated by this strain were also lower than those nodulated by the parental strain. The acetylene reduction capacities of all 3 strains were found to decrease at 25°C relative to 15°C (see Table 7-3). This decrease, however, was much greater for DC21t than DC1t. A higher percentage of nodule bacteria carrying Tn5 at the higher temperature, i.e. fewer 'revertants', might explain this observation.

The mutant strain DC20t had similar acetylene reducing capabilities to the parental strain at 15°C, and none of the nodule isolates were found to carry Tn5. At 25°C acetylene reduction was lower than for the parental strain and a small proportion of the nodule bacteria carried Tn5. Dry weights of plants nodulated with DC20t, however, were considerably lower than those nodulated with DC1t both at 15°C and 25°C. This discrepancy between the acetylene reducing capability of DC20t and the size of its host plant might be explained if the proportion of 'revertants' within the nodule had gradually increased until nitrogenase activity was comparable with that of the parental strain. The contribution of this increased activity to plant growth would take a longer time to become evident.

Large numbers of 'revertants' of DC20t within nodules suggested that this strain might be unstable compared with DC21t for which smaller numbers of revertants were observed. When these strains were streaked three times successively on non-selective TY agar then tested for the retention of kanamycin-resistance, neither were found to have lost the transposon.
Table 7-2. Some symbiotic characteristics of _R. trifolii_ DC1t, DC20t and DC21t nodulating _T. repens_ cv. Huia

<table>
<thead>
<tr>
<th>Inoculum strain</th>
<th>Acetylene reduction; nmoles ethylene formed per plant in 40 hr (a)</th>
<th>Dry weight (mg) (b)</th>
<th>Proportion of bacteria isolated from nodules carrying Tn5 (Km') (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15°C</td>
<td>25°C</td>
<td>15°C</td>
</tr>
<tr>
<td>DC1t</td>
<td>2.1 x 10^3 ± 0.5 x 10^3</td>
<td>7.4 x 10^2 ± 3.7 x 10^2</td>
<td>46.4 ± 7.9</td>
</tr>
<tr>
<td>DC20t</td>
<td>1.6 x 10^3 ± 1.0 x 10^3</td>
<td>2.0 x 10^2 ± 2.9 x 10^2</td>
<td>13.2 ± 8.4</td>
</tr>
<tr>
<td>DC21t</td>
<td>3.9 x 10^2 ± 5.5 x 10^2</td>
<td>3.3 x 10^1 ± 6.0 x 10^1</td>
<td>8.3 ± 4.2</td>
</tr>
<tr>
<td>None: uninoculated plants</td>
<td>0</td>
<td>0</td>
<td>4.4 ± 1.2</td>
</tr>
</tbody>
</table>

(a) Mean value of at least 10 replicates ± 1 standard deviation
(b) Mean value of 16 replicates ± 1 standard deviation, except uninoculated plants - mean of 5 replicates ± 1 standard deviation
(c) Isolates from at least 6 different plants. All isolates tested were rifampicin resistant.
Table 7-3. A summary of the data in Table 7-2

<table>
<thead>
<tr>
<th>Inoculum strain</th>
<th>Acetylene reduction: relative formation of ethylene</th>
<th>Acetylene reduction: ratio of average formation at 25°C to that at 15°C</th>
<th>Relative dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15°C</td>
<td>25°C</td>
<td>15°C</td>
</tr>
<tr>
<td>DC1t</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.5</td>
<td>0.35</td>
</tr>
<tr>
<td>DC20t</td>
<td>0.8 ± 0.5</td>
<td>0.3 ± 0.4</td>
<td>0.12</td>
</tr>
<tr>
<td>DC21t</td>
<td>0.2 ± 0.3</td>
<td>0.04 ± 0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Neither DC20t nor DC21t was auxotrophic and their growth rates were not greatly different from that of the parental strain, insofar as they produced colonies on TY agar after 3 days incubation at 30°C. Both strains nodulated clover plants at the same time as DC11t.

**Mapping of the site of the transposon insertion giving rise to the Eff-2 phenotype**

Preliminary experiments indicated that the transposon insertion was located in either region 6 or 7 of the chromosome of *R. leguminosarum* (see Figure 2-2). Crosses were carried out with *R. leguminosarum* DC11 and DC21 as recipients to ascertain the position of the transposon insertion. The results are given in Tables 7-4 and 7-5, and summarised in Figure 7-1. The transposon insertion was found to map towards the end of section 7 near to section 6. There is the possibility, albeit an unlikely one, that the mutant strain was spontaneous in origin and that the insertion of the transposon was not responsible for the observed phenotype. Testing the effectiveness of strains of *R. leguminosarum*, which had received chromosomal material carrying the inserted transposon, should confirm that this insertion was indeed responsible.

**Isolation of symbiotic mutants: an indirect approach**

Attempts to isolate glutamine-requiring auxotrophs by direct screening or by obtaining D-histidine utilising revertants of a his mutant of *R. trifolii* were unsuccessful.

The first method used for the isolation of MS- and MSX-resistant mutants (see Materials and Methods section) was found to be of no use for the isolation of strains of the latter type. At a concentration of 10mM no resistant mutants were detected even after UV-irradiation. A slightly lower concentration of 5mM was found only to partially inhibit growth of the rhizobia and some colonies were observed to grow above the "background" but
Table 7-4. Analysis of the cross *R. trifolii* rif-1 Eff-2: : Tn5 (Km\(^r\)) (pJB3J1) x *R. leguminosarum* ser-2 ade 88 str-2

<table>
<thead>
<tr>
<th>Selected donor marker</th>
<th>Non-selected markers of recombinants</th>
<th>No. of R(^+) recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ade(^+)</td>
<td>ser(-) Km(^s)</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>ser(-) Km(^r)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>ser(+) Km(^r)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>ser(+) Km(^s)</td>
<td>0</td>
</tr>
</tbody>
</table>

Linkage: ade-88, ser-2 = 1% ade-88, Tn5 (Km\(^r\)) = 4%

Table 7-5. Analysis of the cross *R. trifolii* rif-1 Eff-2: : Tn5 (Km\(^r\)) (pJB3J1) x *R. leguminosarum* ser-2 met/cys-19 str-1

<table>
<thead>
<tr>
<th>Selected donor marker</th>
<th>Non-selected markers of recombinants</th>
<th>No. of R(^+) recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ser(+)</td>
<td>met(-) Km(^s)</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>met(-) Km(^r)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>met(+) Km(^r)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>met(+) Km(^s)</td>
<td>2</td>
</tr>
<tr>
<td>met(+)</td>
<td>ser(-) Km(^s)</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>ser(+) Km(^r)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>ser(+) Km(^s)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>ser(-) Km(^r)</td>
<td>1</td>
</tr>
</tbody>
</table>

Linkage: ser-2, met/cys-19 = 7% ser-2, Tn5 (Km\(^r\)) = 14%

met/cys-19, ser-2 = 9% met/cys-19, Tn5 (Km\(^r\)) = 7%
Figure 7-1. Location of the Tn5 insertion on the chromosome of *R. leguminosarum*. The insertion is assumed to give rise to the Eff-2 phenotype although this has not been confirmed (see text). Numbers on the arrowed lines are the co-inheritance percentages for the alleles shown.
on further testing were found not to be resistant to MSX. A single MS-resistant mutant was obtained after UV mutagenesis of a culture of *R. trifolii* P3. The parental strain used did not carry any identifying genetic markers since the experiment was intended to be of a preliminary nature, but further work did not yield more mutants. It was considered extremely unlikely, however, that the isolate was derived from a contaminating *Rhizobium*, particularly as it was found to be sensitive to the phage Rt1.

The second method involving a gradient plate technique was useful for the isolation of MSX-resistant mutants but no further MS mutants were obtained. A total of six cultures were plated and one resistant mutant from each plate was isolated and purified. All of these mutants were tested on clover plants. The six MSX-resistant mutants were fully effective, and one was found to produce almost twice as much ethylene in the presence of potassium acetate as the wild type (see Table 7-6). They were also tested for their response to the inhibitory effects of ammonium ions when they were 5 weeks old. None were found to reduce significantly more acetylene in the presence of ammonium ions than the wild type as judged on a statistical basis, although strain DC27t reduced significantly less (see Table 7-6).

The MS-resistant *R. trifolii* DC33t was found to be almost completely ineffective (see Table 7-7).

**Discussion**

A direct approach to the isolation of symbiotic mutants yielded six at least partially ineffective strains, two of which were studied further. Although isolated in the same experiment, the different phenotypic characteristics of these two strains strongly suggested that they had arisen independently. In both cases the
Table 7-6. The ammonium inhibition of acetylene reduction by MSX-resistant mutants of *R. trifolii* nodulating *T. repens* cv. Hula

<table>
<thead>
<tr>
<th>Inoculum strain</th>
<th>Acetylene reduction: nmoles ethylene produced per plant in 40 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plants treated with potassium acetate</td>
</tr>
<tr>
<td>DC1t</td>
<td>221.0 ± 52.4</td>
</tr>
<tr>
<td>DC27t</td>
<td>276.4 ± 236.0</td>
</tr>
<tr>
<td>DC28t</td>
<td>273.4 ± 179.0</td>
</tr>
<tr>
<td>DC29t</td>
<td>184.0 ± 67.3</td>
</tr>
<tr>
<td>DC30t</td>
<td>132.1 ± 83.5</td>
</tr>
<tr>
<td>DC31t</td>
<td>196.4 ± 64.7</td>
</tr>
<tr>
<td>DC32t</td>
<td>423.5 ± 41.2 (b)</td>
</tr>
</tbody>
</table>

(a) Significantly different from the wild type at the 10% but not the 5% rejection level.

(b) Significantly different from the wild type at the 1% rejection level.

Table 7-7. Acetylene reduction by *R. trifolii* DC1t or DC33t nodulating *T. repens* cv. Hula

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acetylene reduction: nmoles formed per plant in 40 hr</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC1t</td>
<td>2.5 x 10^3 ± 0.9 x 10^3</td>
<td>23.7 ± 4.8</td>
</tr>
<tr>
<td>DC33t</td>
<td>0.1 ± 0.1</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0</td>
<td>4.2 ± 0.8</td>
</tr>
</tbody>
</table>
defect appeared to affect specifically the ability of the R. trifolii-
T. repens symbiosis to fix nitrogen, although whether this was
the result of a direct mutation involving the nitrogenase complex,
or an indirect one, e.g. affecting energy supply within the
bacteroid, could not be ascertained.

The insertion of Tn5 was considered to be responsible for the
mutant phenotypes observed. The insertion site for Tn5 in
R. trifolii DC21t was found to be chromosomally located.
Similarly, Meade et al. (1979) have located genes involved in
the R. meliloti-alfalfa symbiosis on the bacterial chromosome,
although, as mentioned in the introduction to this chapter,
evidence has suggested that at least some genes involved in
symbiosis are located on a plasmid.

A crude test involving streaking of the mutant strains on non-
selective medium suggested that the transposon insertion in both
was relatively stable. It was still possible, however, that loss
of the transposon was occurring at a low frequency. The
accumulation of "revertants" within nodules observed with both
strains DC20t and DC21t was possibly due to a combination of
loss of the inserted transposon, this loss presumably being an
accurate process in most cases resulting in restoration of the
parental phenotype, and some form of "selection pressure"
imposed by the host plant favouring the establishment of such
non-mutant rhizobia within the host plant. The accumulation
within nodules of revertants of auxotrophs which were symbioti-
cally defective has been observed previously (Ludwig and Signer,

Six independent MSX-resistant strains and one MS-resistant
strain were isolated. It was hoped that such strains would have
alterations to their GS which would also affect its regulatory
activities. The six MSX-resistant mutants were fully effective
and were not derepressed in the presence of ammonium ions.
Brenchley (1973) found that GS was not inhibited by MSX to the same extent as by MS, and an MSX-resistant mutant of R. trifolii was found to have altered levels of GOGAT, but not of GS (O'Gara and Shanmugam, 1977). It is therefore possible that the GS of the MSX-resistant mutants isolated was unaffected by this inhibitor, or that alterations produced did not affect its regulatory abilities.

A mutant resistant to MS, a strong inhibitor of GS, was found to be completely ineffective. This suggests that the rhizobial GS enzyme may be involved in the regulation of nitrogenase activity in the R. trifolii-T. repens symbiosis, as genetic evidence has suggested for other Rhizobium-legume symbioses (Ludwig and Signer, 1977; Kondorosi et al., 1977b). Further biochemical and genetic characterisation would be required, however, to establish that an altered GS was in fact responsible for the phenotype of this mutant.
Chapter 8

GENERAL DISCUSSION
This general discussion is intended to summarise and put into perspective the work described in this thesis.

Genetic studies of *Rhizobium* have mostly been carried out with *R. leguminosarum* (Beringer, 1974; Beringer, Hoggan and Johnston, 1978; Beringer et al., 1978) and *R. meliloti* (Kondorosi et al., 1977a, b; Meade and Signer, 1977; Casadessus and Olivares, 1979), although some studies have included *R. trifolii* (Johnston and Beringer, 1977; Beringer et al., 1978). This thesis reports genetic studies which have been initiated with *R. trifolii* P3 including mutagenesis and the isolation of a range of mutants by conventional procedures and also by transposon mutagenesis. The plasmid R68.45 which has been used for mapping purposes in *R. leguminosarum* (Beringer et al., 1978) and *R. meliloti* (Kondorosi et al., 1977a) was found to have chromosome donor ability in this strain of *R. trifolii*. Since no linkage map of *R. trifolii* had been reported, mapping was carried out on the chromosome of *R. leguminosarum*.

Reports of induced mutagenesis in *Rhizobium* by different mutagenic agents are usually not comparable and sometimes give ambiguous results (see Chapter 1). A comparative study of the effects of a variety of common mutagens showed that only two, MNNG and UV, caused significantly increased frequencies of rifampicin-resistant mutants in a population of *R. trifolii* P3. The highest induced frequency obtained, $5.5 \times 10^{-6}$, following MNNG mutagenesis in TY broth, was still not high when considered in terms of detection of specific non-selectable mutants. This mutagen was capable only of inducing 0.3% auxotrophs in a population of *R. trifolii* P3 as compared with 5% in a population of *R. leguminosarum* 603 (Beringer, Johnston and Wells, 1977). In general, *R. trifolii* P3 was found to be more sensitive than *E. coli* B/r to the lethal effects of mutagens such as EMS and MMS which did not induce mutations in it. This suggested that the bacteria
were killed before they were mutated, so to speak.

Attempts to enhance mutagenesis by chemical treatment or by transfer into *R. trifolii* of plasmids known to do this, were unsuccessful. Attempts to obtain a "mutable" mutant were also unsuccessful, at least from a practical point of view. Although a UV-sensitive mutant of *R. trifolii* which was isolated was considerably more sensitive to the mutagenic effects of UV, it was also more sensitive to its lethal effects so that the maximum yield of mutants induced in this strain was only slightly higher than could be obtained with the wild type. Evidence from the study of this mutant strain and from the work on the efficacy of a variety of mutagens suggested that *R. trifolii*, like *E. coli*, possesses both excision and error-prone pathways of repair, although their contributions to repair and mutagenesis following treatment with different mutagenic agents are certainly different.

The induction of auxotrophs of *R. trifolii* P3 by insertion of a transposon, Tn5, was found to be as efficient as induction of such mutants by MNNG, and the range of these was similar for both methods. The former method had the advantage that otherwise unselectable mutants, once identified, had a selectable phenotypic marker, i.e. kanamycin-resistance. This greatly facilitated the mapping of one of the symbiotic mutants isolated by this method and offered the opportunity of doing likewise for the rest. Meade et al. (1979) were also able to map a number of genes involved in the *Rhizobium*-legume symbiosis following isolation of Tn5-induced symbiotic mutants of *R. meliloti*.

By isolation and characterisation of symbiotic mutants of *Rhizobium*, and mapping of genes involved, it may be possible to identify clusters of genes (operons?) involved in specific stages of the establishment of symbiosis from recognition of the appropriate host plant through to establishment and regulation of nitrogen fixation itself. The distribution of such genes between
the chromosome and cryptic plasmid(s) of the bacteria would also be of interest. Work of this nature, possibly in conjunction with plant genetic studies, would presumably also reveal the contribution of the host plant to the symbiotic association.

As discussed in Chapter 1, the manner in which nitrogen fixation is regulated in the *Rhizobium*-legume symbiosis has not been clarified. The enzyme glutamine synthetase (GS) controls nitrogen fixation in the free-living nitrogen-fixing bacterium *K. pneumoniae*. Genetic evidence has also implicated rhizobial GS in the control of nitrogen fixation in the *Rhizobium*-legume symbiosis (Kondorosi et al., 1977b; Ludwig and Signer, 1977), although biochemical evidence suggests that little, if any, GS is synthesised by bacteroids within nodules.

An ineffective mutant of *R. trifolii* P3 resistant to the glutamate analogue methionine sulphone, a strong inhibitor of GS, was isolated. Although it has not been confirmed that this mutant has an altered GS, it seems likely that it represents a class in which the regulatory, but not catalytic, activity of GS has been altered, such that it "switches off" synthesis of nitrogenase. If this is indeed the case, then the existence of such a mutant gives further support to the hypothesis for the involvement of rhizobial GS in the regulation of nitrogen fixation.

Further genetic studies on the *R. trifolii*-clover symbiosis, complemented by similar studies on other *Rhizobium* spp., should help to elucidate some of the mechanisms involved in the establishment of the *Rhizobium*-legume symbiosis. Such knowledge may be useful in the construction of more efficient inoculant strains of *Rhizobium*, of strains of *Rhizobium* adapted to particular environmental conditions, e.g. low temperature, or possibly ultimately in the construction by genetic manipulation techniques of non-leguminous plants, e.g. cereal crops, capable of fixing nitrogen. These considerations are of increasing
agricultural importance as fertiliser costs rise and the food requirements of an expanding human population grow.
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