Analysis of Ammonium Uptake, a Sigma 54 Regulated Function in *Rhizobium meliloti*.

Thesis
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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Maura Devine Date: 10/8/95

ABSTRACT

Ammonium transport was examined in the symbiotic nitrogen-fixing bacterium *Rhizobium meliloti*. This was done by assaying for the uptake of a radio-labelled analogue of ammonium, ^{14}C-methylamine. The activity was shown to vary depending on the nitrogen source and its concentration in the growth medium, and to be inducible. The effect of various compounds on the actual assay itself was also assessed. Ammonium, methylamine and glutamine were found to inhibit uptake, as did sodium azide, indicating that it was an energy-requiring process. Several mutants in nitrogen assimilation were assayed for uptake, and this showed the activity to be dependent on the sigma factor σ^{54} and its associated activator protein NtrC, but not on the three genes for glutamine synthetase found in *R. meliloti*.

Genes transcribed by σ^{54} show a highly conserved promoter sequence in the -24/-12 region. A primer designed to this sequence allowed use of the PCR technique to amplify fragments containing this sequence from a gene bank of *R. meliloti*. These PCR products were then cloned, and eight transformants with different sized inserts selected for sequencing. Analysis of the sequence data showed one to contain the start of the *nifH* gene, known to be σ^{54}-dependent, and thereby validating this method for the isolation of a group of promoter-specific genes.

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Last but not least - My thanks to my parents for their constant support in whatever I do. It's okay Mum - I've finished being a student now!

This thesis is
dedicated to my parents,
with love and gratitude for
their unfailing support.

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Abbreviations

AAT	aspartate amino transferase
ADH	alaline dehydrogenase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BAP	bacterial alkaline phosphatase
BLAST	basic local alignment search tool
cAMP	cyclic adenosine monophosphate
CAP	catabolite activator protein
CM	chloramphenicol
cpm	counts per minute
Crp	cAMP receptor protein (= CAP)
E	core RNA polymerase
Fnr	anaerobic transcriptional regulator
GABA	γ-aminobutyric acid
GDH	glutamate dehydrogenase
gm	gentamycin
GOGAT	glutamine:2-oxoglutarate aminotransferase
GS	glutamine synthetase
IHF	integration host factor
K_i	inhibitor constant
K_m	Michaelis-Menten constant
km	kanamycin
LSC	liquid scintillation counter
MA	methylamine/methylammonium
MSX	methionine sulphoximine
NAD	nicotinamide adenine dinucleotide
Ntr	nitrogen regulated (system)
ORF	open reading frame
PCR	polymerase chain reaction
P_i	inorganic phosphate
pol II	mammalian RNA polymerase II
P(N)	Poisson value
sm	streptomycin
T_a	annealing temperature
T_m	melting temperature
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Chapter 1 :
Introduction

1.1 FOREWORD

Rhizobium meliloti is a Gram-negative eubacterium, and has many features of its metabolism in common with other members of this general grouping, for example the much-studied enteric organism, *Escherichia coli*. However as a member of the family *Rhizobiaciae*, it also has distinctive characteristics. Three of the five genera in this family - *Rhizobium, Bradyrhizobium* and *Azorhizobium* - are collectively known as the rhizobia, and are distinctive in their ability to fix nitrogen when in an intracellular symbiotic relationship with certain specific plants. They also exist in a free-living form, and can utilise many other nitrogen sources. Members of the *Bradyrhizobium* and *Azorhizobium* genera are also capable of nitrogen fixation in certain free-living conditions.

These two very different modes of existence of which the rhizobia are capable, make them very interesting to study, particularly with regard to the metabolism of nitrogen. On the one hand, in symbiosis, they exist as virtual nitrogen-fixing organelles in the plant cell, whilst on the other, as free-living bacteria, even those capable of non-symbiotic fixation will preferentially scavenge available fixed nitrogen from their environment.

This points to a diversity of metabolic pathways, and more importantly, a complex system of regulation to ensure the efficient use of available nitrogen. The following review describes ammonium assimilation and transport in prokaryotes with an emphasis on the rhizobia, and also the regulatory systems that have been shown to operate in this regard at a genetic level.

1.2 ASSIMILATION OF AMMONIUM[1]

1.2.1 Assimilatory pathways

Ammonia is a key compound in nitrogen metabolism, occurring as it does at a critical juncture between inorganic and organic nitrogen sources. It is an intermediate in nitrate assimilation, the product of dinitrogen fixation and a breakdown product of complex organic nitrogen sources. It is assimilated by bacteria into the amino acids glutamate and glutamine. Glutamate in turn is involved in the synthesis of most other amino acids, whilst glutamine provides nitrogen in the synthesis of purines, pyrimidines, amino sugars, histidine, tryptophan, asparagine, NAD, and *p*-aminobenzoate (Woods and Reid 1993). Aspartate, another important amino acid, can be produced by transamination from glutamate. Two pathways appear to be capable of the incorporation of ammonia into glutamate. The enzyme glutamate dehydrogenase (GDH, E.C.1.4.13) reversibly catalyses the amination of 2-oxoglutarate, using either NADPH or NADH as electron donor:

$$\text{2-Oxoglutarate} + NH_3 + NAD(P)H + H^+ \rightleftharpoons \text{Glutamate} + NAD(P)^+ + H_2O$$

This enzyme can also have a catabolic function, generally associated with the NAD-linked rather than the NADP-linked activity. The K_m for ammonia is usually high, indicating that it operates efficiently only at high ammonia concentrations. However most bacteria appear to have an alternative mechanism of achieving the same overall result involving two enzymes: glutamine synthetase and glutamate synthase. Glutamine synthethase (GS, E.C.6.3.1.2) catalyses the following (in the presence of divalent metal ions):

$$NH_3 + \text{L-Glutamate} + ATP \rightarrow \text{L-Glutamine} + ADP + P_i$$

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[1] As is the convention in the literature, the terms ammonia and ammonium are used without specifying the state of protonation. When this is required, chemical formulae are used, i e NH_3, NH_4^+

Glutamate synthase (glutamine : 2-oxoglutarate aminotransferase, GOGAT, E.C.2.6.1.53) catalyses the following:

L-Glutamine + 2-Oxoglutarate + NAD(P)H + H^+ →

L-Glutamate + L-Glutamate + $NAD(P)^+$ + H_2O

These two reactions have the same overall effect as GDH, but are irreversible, and expend one mole of ATP per mole of ammonia assimilated. This system is able to handle lower concentrations of ammonia than GDH.
Another important amino acid is aspartate. It can be produced from glutamate by aspartate aminotransferase (AAT, E.C.2.6.1.1) as follows.

L-Glutamate + Oxaloacetate ⇌ L-Aspartate + α-Ketoglutarate

This reaction can also be catalysed by broad specificity amino transferases (e.g. by several of the aromatic amino transferases in *Rhizobium meliloti*, [Kittell *et al.* 1989]). Another possible assimilatory mechanism has also been suggested, that of alanine dehydrogenase (ADH, E.C.1.4.1.1) (Miller *et al.* 1991). This catalyses the following:

Pyruvate + NADH + NH_4^+ + H^+ ⇌ L-Alanine + NAD + H_2O

As the equation shows, this is an NADH-linked activity, and the reaction is reversible.

1.2.2 Ammonium assimilation in *E. coli*

1.2.2.1 Glutamate dehydrogenase

Enteric bacteria have been reported to possess only the NADPH-dependent GDH, an enzyme of 300 kDa, consisting of six identical subunits, with a K_m for ammonia of approximately 1 mM (Merrick, 1988). The gene has been isolated (*gdhA*), and shows strong homology to that of other bacteria, and even to eukaryotic GDHs. In the related organisms *Klebsilla aerogenes* and *Klebsiella pneumoniae*, the level of GDH is low in N-deficient medium, and high for growth on a high ammonia concentration. In *K. aerogenes*, this is due to the repression of transcription of *gdhA* (during ammonium limitation) by the Nac protein, an activator / repressor associated with the Ntr system (See Section 1.4) (Kennedy *et al.* 1994). This supports the idea that GDH is only of assimilatory significance when there is a high concentration of available ammonium .

1.2.2.2 GOGAT

The GOGAT enzyme in *E. coli* has been characterized as an iron sulphur protein consisting of 8 subunits, 4 of a molecular weight of between 135 - 175 kDa, and 4 of 53 kDa. It is encoded by the *gltB* operon, the larger subunit gene being transcribed first. Mutants in *gltB* are unable to grow on low ammonium concentrations, but there is no evidence of the nitrogen status of the cell regulating levels of this enzyme (Merrick 1988).

1.1.1.3 Glutamine synthetase

Control of GS in enteric bacteria (GSI) is a complex system and has been extensively studied (Reviews: Brown 1980, Magasinik 1982). The enzyme itself consists of 12 identical

subunits of approximately 55 kDa molecular weight each. They are arranged in two hexagons and stabilised by divalent cations. Each subunit contains a specific tyrosyl residue (number 397) which can be adenylylated enzymatically to modify the enzyme activity. The complete molecule can therefore contain from 0 to 12 adenyl residues, the latter case resulting in complete inactivation of its biosynthetic activity, whilst partial adenylylation makes it more sensitive to feedback inhibition by products of glutamine metabolism. There are believed to be binding sites on each subunit for these inhibitors, and also for certain amino acids. In addition to the biosynthetic reaction described above, GS is also capable of catalysing a transferase reaction:

L-Glutamine + Hydroxylamine → γ-Glutamyl hydroxamate + NH_3

At pH 7.15, (known as the isoactivity point for the organism) transferase activities for adenylylated and unadenylylated forms of the enzyme are equal in the presence of Mn^{2+}. However Mg^{2+} will activate only the unadenylylated form, and inhibits the adenylylated if Mn^{2+} is also present. This therefore gives a means of calculating the total enzyme activity and extent of adenylylation for different conditions.

Both adenylylation and deadenylylation are catalysed by the same enzyme, adenylyltransferase (ATase), whose activity is controlled by a regulatory protein, P_{II}. In its unmodified form, P_{II} stimulates adenylylation. However it is also subject itself to modification, by a uridylyltransferase (UTase) capable of sensing the nitrogen status of the cell in the form of the glutamine : α-ketoglutarate ratio. Uridylylation of P_{II} to P_{II}-UMP (using UTP) occurs in ammonia-poor conditions, and in this form the regulator stimulates deadenylation by ATase, and hence increased GS activity. UTase can also catalyse the reverse reaction (i.e. removal of the uridylyl group) when the glutamine :

α-ketoglutarate ratio is high. (Hence it is also designated UR : uridylyl-removing enzyme.)

E. coli GS is also subject to control on a genetic level, as shown by its repression for growth on good nitrogen sources or high ammonium concentration, and its derepression in conditions of ammonia limitation. Therefore it is under "nitrogen control", as are several other genes involved in nitrogen utilisation, e.g. those for histidine and for nitrate utilization. These have been shown to be under the control of the Nitrogen Regulatory (Ntr) system (See Section 1.4). The structural gene for GS is designated *glnA*, and exists as part of the *glnALG* operon, *glnL* and *glnG* encoding two proteins of the Ntr system, NtrB and NtrC respectively (also called NR_{II} and NR_{I}). GS was once hypothesized to have regulatory functions itself, but this was subsequently shown to be due to effects on the operon as a whole (Ludwig and Signer 1977, Brown 1980, Magasinik 1982). The transcription of *glnA* is dependent on the sigma factor σ^{54} (as are all Ntr-controlled genes), encoded by *rpoN (ntrA*, originally designated *glnF* in *E. coli*), and on one of its specific activator proteins, NtrC. NtrB also has a controlling effect on GS as it phosphorylates NtrC, activating it, and P_{II}-UMP is also involved (to be discussed more fully later). Other relevent genes are *glnB*, whose product is the P_{II} regulator protein, *glnD*, the gene for UTase, and *glnE*, which codes for the adenylyltransferase. Figure 1.1 summarises the regulation of *E. coli GS*.

GS, GOGAT and GDH are not thought to be genetically linked in *E. coli*, but in *K. aerogenes*, GOGAT has been suggested to affect the synthesis of GS, which in turn may influence GDH synthesis (Brown 1980).

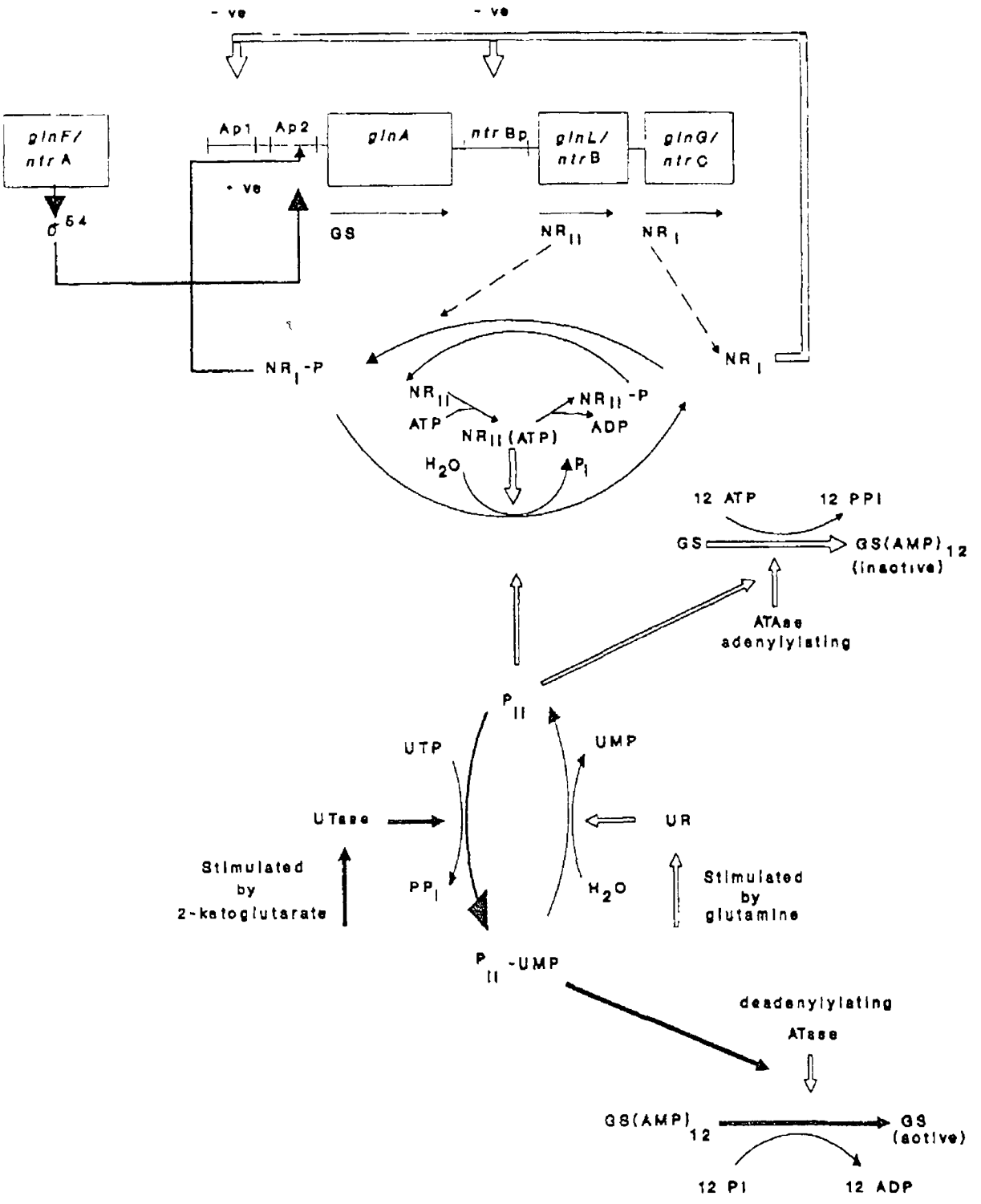


Figure 1.1 : Regulation of GS in *E. coli*. The pathways described in the text for the covalent modification and transcriptional regulation of GS are shown. (Taken from Woods and Reid 1993).

1.2.3 Ammonium assimilation in the *Rhizobiaciae*

1.2.3.1 Glutamate dehydrogenase (GDH)

The GDH enzyme is not universally found in bacteria, especially in its assimilatory (usually NADP-linked) form. Brown and Dilworth (1975) detected NAD-linked GDH activity in *Rhizobium leguminosarum, Rhizobium lupini, Rhizobium meliloti, Rhizobium phaseoli, Rhizobium trifolii* and *Bradyrhizobium japonicum*. However, the NADP-linked activity was absent in the bacteroids of *R. leguminosarum* and *R. lupini*, where only NAD-linked activity was detected. In free-living *Rhizobium* sp. 32H1, GDH showed only catabolic NAD-linked activity, and *glt* mutants (lacking GOGAT) could not utilise ammonium (Ludwig 1980). In contrast, Osburne and Signer (1980) found only assimilatory GDH in *R. meliloti* 2011, but found it insufficient for growth on ammonium by a GOGAT-deficient mutant, even at a concentration of 100 mM. Ali *et al* (1981) found differences between strains of *R. meliloti*, some lacking assimilatory GDH altogether whilst strain 2011 had NADH-linked and NAD-linked activities that were both assimilatory. In contrast to the previous study, a GOGAT-deficient mutant could grow on ammonium (presumably by using GDH), but needed higher ammonium concentrations than the wild type. Other studies have shown the lack of GDH in strains of *B. japonicum* (O'Gara *et al* 1984), *Bradyrhizobium* sp. ANU289 (Howitt and Gresshoff 1985), and *R. phaseoli* (Bravo and Moro 1988). Introduction of the *E. coli gdh* gene into strains lacking GDH was shown to compensate for GOGAT deficiency caused by mutation or inhibition (i.e. to allow ammonium assimilation), but appeared to interfere with nitrogen fixation in symbiosis (Lane *et al* 1986, Bravo *et al* 1988).

1.2.3.2 GOGAT

As the studies cited above (and others, e.g. Osburne 1982, Donald and Ludwig 1984) suggest, the GS-GOGAT pathway appears to be the major route (and in many cases the only one) of ammonium assimilation in Rhizobia. GOGAT mutants have been shown to lack assimilation of ammonia (Osburne and Signer 1980, O'Gara *et al.* 1984), except in the one case already cited (Ali *et al.* 1981). As with GDH, GOGAT activity is linked to either NAD or NADP, the specific co-enzyme seeming to depend on the genus, i.e. *Rhizobium* strains appear to have the NADP-linked form, and *Bradyrhizobium* strains the NAD-linked activity (Brown and Dilworth 1975, Ali *et al.* 1981). *Rhizobium* sp. ORS571 (subsequently renamed *Azorhizobium caulinodans*) showed both activities, but mutants in each showed different nitrogen fixation abilities (both in culture and in planta). The mutants lacking the NAD-GOGAT activity were Nif^+, but those deficient in the NADP-linked activity were unable to fix nitrogen (Donald and Ludwig 1984).

1.2.3.3 Glutamine synthetase

The family *Rhizobiaciae* has shown itself to be very different from other bacteria in that many of its members possess more than one GS, each with similar affinities for ammonium, but different systems of regulation (Fuchs and Keister 1980, Woods and Reid 1993, Brown *et al.* 1994).

Darrow and Knotts (1977) first showed the existence of two GS enzymes in *Bradyrhizobium japonicum*, one similar to enteric GS and the other heat labile and apparently not subject to adenylylation. Ludwig (1980) showed that *Rhizobium* sp. 32H1 (an unusually poor user of ammonium) utilised the GS-GOGAT pathway, but its GS activity was easily repressed by even low concentrations (1 mM) of ammonium. He identified two forms of GS present, one derepressed by the absence of ammonia by more than 50 fold,

the other by only 2 fold. The latter however was subject to adenylylation in the same manner as enteric GS, and seemed to be the major contributor to ammonia assimilation (GSI). The more repressible activity (GSII) was distinguishable by PAGE and also by its heat labile nature. The author suggested that while a true GS, it is possibly involved in supplying glutamine to the amidotransferases in the purine pathway, rather than towards glutamate synthesis. Fuchs and Keister (1980) examined the GS enzymes of several *Rhizobium, Bradyrhizobium* and *Agrobacterium* species, (three genera in the family *Rhizobiaciae*). All 12 strains tested were found to contain both GSI and GSII, in contrast to 11 strains from various other genera which contained only GSI. There were some differences in the relative heat stabilities and isoactivity points of strains, but nevertheless each strain had a GSI that was heat stable and subject to adenylylation, and a GSII that was heat labile and subject to repression. Only *Azorhizobium caulinodans* was shown to have a single GS activity, corresponding to GSI (Donald and Ludwig 1984). However, as for other *Rhizobiaciae*, this was expressed constitutively and subject to regulation by adenylylation. More recent work has shown by mutational analysis that the two GS activities are definitely encoded by separate genes, and the genes for each have been cloned and in some cases sequenced (Somerville and Kahn 1983, Carlson *et al.* 1985, Carlson and Chelm 1986, Filser *et al.* 1986, Shatters *et al.* 1989, Moreno *et al.* 1991, Patriarca *et al.* 1992).

1.2.3.3.1 Glutamine Synthetase I

GSI is similar in structure to the enteric GS, consisting of 12 subunits of approximately 52 - 59 kDa each, and also showing high sequence homology to the enteric protein (Espín *et al.* 1994). Unlike the *E. coli* enzyme, its synthesis is not significantly regulated by the nitrogen source and it does not have a σ^{54}-dependent promoter - i.e. is not under direct Ntr control (Carlson *et al.* 1987,

Shatters *et al.* 1989). However Chiurazzi and Iaccarino (1990) showed that *R. leguminosarum* bv *viciae* has a *glnB* gene upstream of *glnA* (the GSI gene) and that these two genes are often transcribed together, *glnB* being preceeded by a σ^{54}-dependent promoter. There is also a promoter preceeding the actual *glnA* gene that is not subject to nitrogen regulation, but overall there appears to be a slight nitrogen-dependent effect on transcription. There also is in *R. etli* (Moreno *et al* 1991), namely a 1 fold increase in levels in glutamate over ammonia. Carlson *et al.* (1987) also reported only slight regulation of *glnA* transcription in *B. japonicum*, while in *R. meliloti glnA* transcription has been shown to be constitutive (Szeto *et al.* 1987, de Bruijn *et al.* 1989). As in *E. coli*, the main mechanism of GSI regulation seems to be via adenylylation of the enzyme (although activity in the adenylylated as well as the unadenylylated form has been reported in *Bradyrhizobium* sp. ANU289 [Howitt and Gresshoff 1985]). The details of the mechanism are unknown, although NtrC and P_{II} may be involved (Espín *et al.* 1994). In the study by Ludwig (1980), GSI showed other differences from the GS in *E. coli* in that its unadenylylated form had the same pH profile as adenylated enteric GS, and it also had a lower ratio of biosynthetic to transferase activity.

1.2.3.3.2 Glutamine Synthetase II

GSII is distinct from all other prokaryotic glutamine synthetases. It has been characterized as being composed of eight subunits of 36 - 42 kDa each, arranged in two discs of four. It is heat labile, and does not appear to be regulated by adenylylation. [Note: Regulation by adenylylation is also not found in the GS enzymes of *Bacillus* and *Clostridium* species, but they still belong structurally to a subgroup of the GSI grouping (Woods and Reid 1993).]

Its transcription shows the characteristics of Ntr control: e.g. in *B. japonicum* a 20 fold induction was demonstrated

in response to the nitrogen source (Carlson *et al.* 1987). Also σ^{54}-dependent promoter sequences have been found upstream from the structural gene (*glnII*), and an enhancing effect of NtrC demonstrated (Martin *et al.* 1988, Shatters *et al.* 1989, Moreno *et al.* 1992, Patriarca *et al.* 1992). Patriarca *et al.* (1992) showed that binding of NtrC to an upstream activation site was essential for transcription of the gene. *B. japonicum glnII* also appears to be activated by NifA (a transcription activator associated with symbiosis) during symbiosis and under conditions of microaerobiosis (Martin *et al.* 1988, Martin and Chelm, 1991). (This organism is also the only one that appears to require GSI or GSII for symbiosis, demonstrated using a *glnA glnII* double mutant.) In *R. phaseoli*, GSII is repressed by ammonium and glutamine and derepressed by glutamate and nitrate. However an increase in the glutamine to α-ketoglutarate ratio results in the adenylation of GSI and the induction of GSII, but if this is very rapid ("ammonium shock") there is inactivation of GSII also (Bravo and Mora 1988). Manco *et al.* (1992) also observed this for *R. leguminosarum*, and inferred some type of post-translational modification taking place, causing a rapid decrease in transferase activity. However, they showed that biosynthetic activity was not affected in the same way - i.e. there was a dissociation of the two activities. They also showed the existance of two isoforms of GSII with similar molecular weights but different isoelectric points, and that the octameric enzyme could dissociate into tetramers; but neither phenomenon was shown to regulate activity.

The sequenced *glnII* gene shows homology to eukaryotic GS, and this combined with its presence apparently exclusively in plant symbionts/pathogens (it has also been found in *Frankia* species, [Rochefort and Benson 1990]) led to suggestions of lateral transfer from plants (Carlson and Chelm 1986). However this theory has now been discounted, as phylogenetic analyses show that the bacterial sequence diverged from the eukaryotic sequence prior to the

separation of plants from animals (Somerville *et al.* 1989, Patriaca *et al.* 1992, Brown *et al.* 1994), and also since GSII has been found in free-living *Streptomyces* (Woods and Reid 1993).

1.2.3.3.3 Glutamine Synthetase III

Work with *R. meliloti* mutants in *glnA* and *glnII* prompted speculation that a third GS enzyme might exist, one having biosynthetic but not transferase activity (Somerville *et al.* 1989). Kumar and Rao (1986) had previously described three chromatographically distinct species in rhizobia, also distinguishing them by sensitivity to high temperature and inhibitors. *R. meliloti, R. leguminosarum* and *R. phaseoli* were each shown to have substantial proportions of the three forms, unlike eukaryotes which were shown to have only one form. However both forms I and II were also found in *E. coli*, a finding not consistent with present knowledge of enteric glutamine synthetase. There have also been reports of three forms of GS in *R. lupini* bacteroids, although only two forms in free-living culture (Kaush *et al.* 1984, Tsuprun *et al.* 1987). GSIII is described as structurally similar to GSI (a 600 kDa protein made up of 12 monomers) and also subject to adenylylation. The first genetic evidence of a third GS came when *R. meliloti* was shown to contain a third locus capable of complementing glutamine auxotrophy in *E. coli* (de Bruijn *et al.* 1989). This was designated *glnT*, and shown to be an operon coding for five polypeptides, with no homology to *glnA* or *glnII*. Like *glnII*, the operon was transcribed under Ntr control, but no product was detected in *R. meliloti*, and an *R. meliloti glnAglnII* double mutant was unable to assimilate ammonia. However Shatters *et al.* (1993) have been able to characterize a third GS enzyme in another strain of *R. meliloti* (104A14 instead of 1021). A *glnAglnII* double mutant of this strain could assimilate ammonium, and was found to do so by producing substantial amounts of an octameric enzyme consisting of subunits of 46.5 and 49 kDa

(one possibly being a modified form of the other). Unlike GSI and GSII, this enzyme showed a very low transferase activity, but high biosynthetic activity. (This is also different from the third GS enzymes previously described by Kumar and Rao [1986] and Kaush *et al.* [1984]). The K_m values for ammonium and glutamate were high compared to the other GS enzymes, but like them, it required glutamate, ammonium, ATP and a divalent cation for activity. It was also less sensitive to various inhibitors and more heat stable, but nevertheless appeared to be a true GS, rather than a protein showing some secondary activity for glutamine synthesis. The authors also showed GSIII to be the product of the *glnT* locus previously identified by de Bruijn *et al.* (1989), and that it was present in bacteroids formed by the double mutant strain. However there was no evidence of its presence in wild type or single mutant free-living or symbiotic cells, suggesting its repression by active GSI and GSII.

In addition to control of its transcription (by the Ntr system), there is also evidence for post-translational modification of GSIII (Liu and Kahn 1995). This is not by adenylylation, but by ADP-ribosylation of an arginine residue. This has been carried out *in vivo*, and appears to inhibit enzyme activity. The same type of modification is known to affect the GS of *Rhodospirillum rubrum*, as also does adenylylation (Woehle *et al.* 1990).

A *glnT* gene has also been identified in *R. etli*, and been shown to encode GSIII (Chiurazzi *et al.* 1992). It was shown to have similar properties in terms of biosynthetic / transferase activities and to be absent in the wild type as was the *R. meliloti* enzyme. The structural gene for GSIII was narrowed down to a 1.3 kilobase open reading frame (ORF), encoding a protein of a predicted molecular mass of 48 kDa (435 amino acids). Another ORF in the operon showed homology to a *B. subtilis* gene involved in purine biosynthesis, suggesting a role for GSIII in this pathway.

[Note: Woods and Reid (1993) also refer to "GSIII" in two anaerobic rumen bacteria (with no reference to that in Rhizobia). However this appears to be a totally different enzyme to the *glnT* product, consisting as it does of a hexamer with subunits of 729 amino acids and molecular weight 82.8 kDa each (Goodman and Woods 1993).]

1.2.3.4 Ammonium assimilation in bacteroids

Little is known about the assimilation of ammonia by rhizobia when they are in their bacteroid state in plant root nodules. It appears that the bulk of the ammonia synthesized by the nitrogenase enzyme is exported to the plant cytosol, and assimilated by plant enzymes. The chief assimilatory pathway in plants is also via GS and GOGAT (Ohyama and Kumazawa 1980, Ta *et al.* 1986, Robinson *et al.* 1991, Baron *et al.* 1994), and levels of these enzymes have been shown to increase in the cytosolic rather than the bacteroid fraction of the nodule in response to nodule development and nitrogen fixation (Scott *et al.* 1976, Hirel *et al.* 1987).

Glutamate appears to be an important compound in *B. japonicum* bacteroids, being the ultimate recipient of most of the carbon supplied as malate, and forming a large pool in the bacteroids (Streeter 1987, Salminen and Streeter 1987, 1990, Streeter and Salminen 1990). Alanine, glutamine and γ-aminobutyric acid (GABA) are also major bacteroid nitrogenous compounds (Miller *et al.* 1991) and this has led to suggestions of ADH and GDH being important assimilatory enzymes in symbiosis, and also of the enzymes of the GABA shunt being necessary for fixation. Miller *et al.* (1991) suggested that *R. meliloti* is unique in having high GDH activity in the bacteroid, and that this accounts for an accumulation of glutamate and hence GABA (also unusually high in *R. meliloti* bacteroids). Kouchi *et al.* (1991) also suggested a role for GDH in *B. japonicum* bacteroids, but given that strains lacking this enzyme are unaffected symbiotically, it seems highly unlikely that it is

essential. A role for ADH has also been suggested in rhizobial ammonium assimilation in bacteroids (Ohyama *et al.* 1980, Miller *et al.* 1991, Kouchi *et al.* 1991).

However strains lacking assimilatory GDH in their free-living state are still capable of symbiosis and fixation. Also while a GOGAT-negative mutant of *R. meliloti* was unaffected symbiotically, a revertant with unusually high GDH activity could not fix nitrogen (Osburne and Signer 1980). In *B. japonicum*, a GOGAT-negative mutant (possessing no GDH in the wild type) was unable to fix nitrogen symbiotically (although it could ex planta); complementation with the *E. coli gdhA* gene did not remedy this (O'Gara *et al.* 1984, Lane *et al.* 1988). Also the expression of *E.coli* GDH in *R. phaseoli* appeared to interfere with fixation (Bravo *et al.* 1988). GS mutants of *R. meliloti* have also been shown to be unaffected symbiotically, including a *glnAglnII* double mutant (de Bruijn *et al.* 1989), but another double mutant was been shown to express GSIII in the nodule (Shatters *et al.* 1993), so a role for assimilatory GS cannot be totally ruled out. Similar double mutants of *B. japonicum* do show an inability to nodulate (Carlson *et al.* 1987) and it has been suggested that this is due to a lack of GSIII in this organism (Shatters *et al.* 1993). Martin *et al.* (1988) also gave evidence of *nifA*-dependent transcription of *B. japonicum glnII* during symbiosis, so a symbiotic role is possible, at least in this particular strain. Tsuprun *et al.* (1987) found evidence of all three GS enzymes in bacteroids of *R. lupini*, but activities were low for GSI and GSIII. However, not only were GSII levels higher, but there did not seem to be the same repressive effect of high ammonium levels as found for free-living cultures. This points again to another possible route of *glnII* expression in symbiosis, and a role for GS activity in bacteroid ammonium assimilation.

Aspartate amino transferase activity has also been shown to be essential for symbiosis by *R. meliloti* (Rastogi and

Watson, 1991). This has a role in supplying aspartate for proteins, but since aromatic amino transferases can also catalyse that reaction (Kittell *et al.* 1989), a more important role for this enzyme has been suggested, namely in the channelling of aspartate into the TCA cycle. Rastogi and Watson (1991) suggested that the dicarboxylic acid transport system (known to be essential for symbiosis) is actually used for the importation of aspartate to the bacteroids. This is implying that the plant provides a fixed nitrogen source to the bacteroids, so possibly *R. meliloti* does not require its own usual assimilatory paths (GS/GOGAT or GDH) at all when in symbiosis.

1.3 THE TRANSPORT OF AMMONIUM

1.3.1 Active versus passive transport

Ammonia, as well as being an important intermediary in nitrogen assimilation in bacteria, can also be supplied in the environment itself, and is often used preferentially to another nitrogen source such as nitrate (Boussiba and Gibson 1991). In that case, its passage into the cell becomes the first step in its assimilation. Ammonia in its non-protonated form (NH_3) can diffuse readily through biological membranes (Kleiner 1985), so the idea of a specific transport system for it was initially regarded with scepticism. Certainly, diffusion alone can provide sufficient ammonium for a microorganism provided the concentration gradient is steep enough, i.e. when there is a high external NH_3 concentration and rapid internal assimilation. However, the non-protonated form exists in equilibrium with the protonated form (NH_4^+), according to the equation :

$$NH_3 + H^+ \rightleftharpoons NH_4^+ \; ; \qquad pK_a = 9.25$$

Therefore the ratio of diffusible ammonia to the ammonium ion is dependent on pH: the higher the pH, the higher the proportion of NH_3 to NH_4^+. For alkalophilic organisms at least, a high ammonia environment seem to provide ample nitrogen without any need for active transport (Boussiba and Gibson 1991). However for low external ammonia concentrations the available diffusible ammonia is in very short supply and active transport systems have been shown to operate for the uptake of the protonated form, ammonium.

1.3.2 Assaying for ammonium transport

Various methods have been used to identify putative ammonium transport systems in both eukaryotic and prokaryotic organisms. These can generally be divided into

firstly, assays of ammonium loss from the surrounding media, and secondly, assays of the intercellular accumulation of a radiolabelled analogue of ammonium, ^{14}C-methylamine. Also the presence of a high concentration of ammonium within cells compared to a low extracellular concentration is seen as pointing towards an uptake system.

1.3.3 Incidence of transporters

Kleiner's review in 1985 listed over 30 bacterial ammonium transporters, mostly investigated by measuring methylammonium uptake. The species ranged from *E. coli*, *K. pneumoniae*, *Azotobacter vinelandii*, *Azospirillum*, *Rhizobium* and *Rhodospirillum* strains, to *Clostridium*, *Frankia* and several cyanobacteria. Since then, further work has been done on rhizobia, and on several different cyanobacteria (review by Boussiba and Gibson 1991). The *E. coli* system has also been further characterised, and attempts made to clone and sequence the responsible gene (Jayakumar *et al.* 1989, Fabiny *et al.* 1991). However this appears to have been unsuccessful (Neuwald *et al.* 1992). To date the only sequenced ammonium transporter genes are both from eukaryotes - *Saccharomyces cerevisiae* (Marini *et al.* 1994) and *Arabidopsis thaliana* (Ninnemann *et al.* 1994), but interestingly both share sequence homology with a *Bacillus subtilis* gene (*nrgA*) known to be nitrogen regulated and encoding a hydrophobic protein (Atkinson and Fisher 1991).

1.3.3.1 Gram-negative eubacteria (non N_2-fixing): *E.coli* and others

Ammonium uptake by *E. coli* has been studied using the analogue methylamine. Initial reports indicated the ability of cells to maintain a 100 fold gradient of the analogue, and that this activity was inhibited by ammonium but not glutamate (Stevenson and Silver, 1977). More recent studies have also shown ammonium to inhibit competitively,

(suggesting it is the natural substrate of the transporter), and that it also represses the activity (Servín-González and Bastarrachea 1984, Jayakumar *et al.* 1985). The latter group further characterized the system as an ammonium (methylammonium) / potassium antiport requiring both ATP and an electrochemical H^+ gradient. Activity was reduced drastically by osmotic shock (92%), but not due to the loss of periplasmic binding proteins, as it could be regained by incubation with glucose and K^+. Growth on 20 mM glutamate allowed expression of transport, whilst 1 mM NH_4^+ was sufficient to prevent it. A K_m of 36 μM was calculated for methylammonium, and a K_i of 0.51 μM for ammonium.

Regulation of the *E. coli* transporter has been linked to the Ntr (nitrogen regulated) system in two studies. The activation of ammonium transport in cells that are transferred from low to high nitrogen medium, and the ability of chloramphenicol (an inhibitor of protein synthesis) to inhibit this (Servín-González and Bastarrachea 1984), suggests a gene under "nitrogen control". This has been further studied using mutants in the *gln* operon (previous reference and also Jayakumar *et al.* 1986). These studies show a requirement for NtrC and NtrA in the expression of ammonium transport, and NtrB in its repression. The product of *glnA*, glutamine synthetase, is not itself required, and the lack of transport noted in some *glnA* mutants is ascribed to polar effects on the operon. Servín-González and Bastarrachea also noted that glutamine had an inhibitory effect on the assay, equivalent to that of ammonium, but nevertheless cells grown on glutamine (6.8 mM) did exhibit transport activity, though to a lesser level than those grown on low (0.5 mM) ammonium. (Jayakumar *et al.* found 10 mM glutamine for growth gave 65% of the activity when 20 mM glutamate was used.) The inhibition was not due to competition for a glutamine transporter. Also mutants lacking in GOGAT (*gltB* mutants), which probably have high intracellular concentrations of glutamine and are known to be Ntr-negative, were unable to express methylammonium transport.

Another example of a non nitrogen-fixing Gram negative eubacteria in which ammonium transport has been studied is *Alcaligenes eutrophus* (Jahns *et al.* 1986). Radiolabelled methylammonium can be taken up by this organism to build up a 200 fold gradient. The uptake is energy-dependent, inhibited by ammonium (to which the carrier shows a higher affinity), but not susceptible to osmotic shock. Higher rates in conditions of nitrogen deficiency or when grown on poor nitrogen sources (e.g. nitrate) again suggested a carrier under "nitrogen control". A K_m value of 35 - 111 μM was calculated for methylammonium, and a K_i for ammonium of < 20 μM.

1.3.3.2 Ammonium transport in nitrogen fixers

Interest in ammonium transport in nitrogen fixers initially stemmed from the powerful repressive effect ammonium is known to have on nitrogenase (Laane *et al.* 1980). Also the idea of isolating or constructing mutants of free-living fixers that would excrete fixed nitrogen rather than take it up was attractive from an agricultural point of view.

1.3.3.2.1 Free-living fixers

Clostridium pasteurianum is one of the few Gram-positive eubacteria in which ammonium transport has been studied. Kleiner and Fitzke (1981) showed that it could build up a 100 fold gradient of methylammonium, but had a greater affinity for ammonium. However more attention has been paid to the Gram-negatives, particularly *K. pneumoniae* and *A. vinelandii*.

Kleiner (1976) showed that *K. pneumoniae* could maintain a 100 fold concentration gradient of ammonium across its cell membrane. Later work using methylammonium showed its ability to build up an 80 fold gradient of the analogue, and that this uptake was subject to repression by ammonium.

A K_m of 140 μM for MA and a K_i of 7 μM for ammonium were calculated (Kleiner 1982). A mutant of *K. pneumoniae* deficient in ammonium uptake was isolated by its inability to grow on 1 mM NH_4^+ (Castorph and Kleiner 1984). The mutant lacked the ability to transport ^{14}C-methylamine, but still contained the same ammonium assimilation enzymes as the wild type (GS, GOGAT and GDH), although at different levels. However the possibility remains that this may be a regulatory rather than a structural mutant (Jayakumar *et al.* 1989). Glutamine and its analogue methionine sulfoximine (MSX) were both shown to inhibit MA uptake in this organism (Kleiner and Castorph 1982), and the involvement of the Ntr system in its regulation has also been indicated (Kleiner 1982).

A. vinelandii was also shown to be able to maintain a 100 fold concentration gradient of ammonium across its cell membrane (Kleiner 1975), and uptake was further investigated both by measuring depletion of ammonium from the surrounding medium and by assaying for accumulation of radiolabelled methylammonium (Barnes and Zimniak 1981, Gordon and Moore 1981). Again, ammonium was found to competitively inhibit MA uptake (K_i calculated in the two studies as 1 μM and 3 μM respectively). Barnes and Zimniak also found inhibition by uncouplers (suggesting involvement of the electrochemical gradient), and calculated the K_m for MA as 25 μM. Amino acids were found not to be inhibitory - including asparagine and glutamine. Gordon and Moore also found arsenate to inhibit (Barnes and Zimniak did not), and other indicators of ATP involvement. Their results also gave evidence for the possible existence of two MA uptake systems (A Lineweaver-Burk plot of MA uptake appeared biphasic). The calculated K_m's were 61 μM and 661 μM. Jayakumar and Barnes (1984) also investigated ^{14}C-methylammonium uptake by *A. vinelandii*. They found that growth in the presence of ammonium, methylammonium, glutamine or asparagine significantly decreased the transport activity. The recovery of activity by cells previously incubated with ammonium could be effected by

transfer to nitrogen-free medium, but unlike in *E. coli*, this was not inhibited by chloramphenicol. Several glutamine analogues also inhibited transport, only one of which (MSX) was also a GS inhibitor. Their results point to independent regulation of transport and of GS. The regulation of transport appears to be by inhibition of the transporter by the assimilatory products (via GS) of ammonium and MA (glutamine and γ-*N*-methylglutamine respectively) rather than by repression of gene expression.

The phototrophic bacterium *Rhodospirillum rubrum* has also been shown to accumulate methylamine against a 200 fold gradient when grown on glutamate or aspartate but not ammonium (Alef and Kleiner 1982). Activity was inhibited by KCN, azide and ammonium, the latter being able to cause efflux of ^{14}C-MA. The K_m for MA was calculated at 110 μM, with a K_i of 7 μM for ammonium.

Ammonium uptake was examined in *Rhodopseudomonas capsulata* using an electrode and the indophenol assay to follow loss of ammonium from the medium (Genthner and Wall 1985). Kinetic analysis appeared to show two systems with K_m's of 1.7 μM and 11.1 μM. Growth on glutamine did not significantly affect uptake. A mutant lacking GS activity (and appearing to posess an inactive form of the enzyme) was also deficient in uptake, but both functions could be restored by the introduction of a plasmid carrying the *glnA* gene by conjugation. However the resulting uptake activity tended to be less than the wild type while GS activity was 4 - 5 times greater. The authors interpret their results to show "tight coupling" of transport and assimilation rather than simply "diffusion pulled by assimilation".

Rhodobacter sphaeroides another purple non-sulphur bacterium, also showed uptake of ammonium and of methylammonium (Cordts and Gibson 1987). The uptake of MA appeared to be biphasic, with the second slower phase linked to assimilation. MA uptake was dependent on the

growth condition of the cells, being repressed by ammonium, and was also inhibited by ammonium in the assay, its addition causing efflux of MA from cells (K_i < 1 μM). Ammonium uptake still took place in the absence of MA uptake (e.g. growth on ammonium) indicating a second carrier. Both required energy in the form of proton motive force, not ATP, and there was not a K^+/MA antiport as described for *E. coli*. By monitoring the intracellular pools of ammonium and MA, transport and assimilation were shown to be separate processes and steady pools were maintained even when there was inhibition by MSX, and in GS mutants. (The absence of transport in GS mutants was explained by the net intracellular production of NH_4^+ from amino acid and protein turnover being sufficient to maintain the intracellular pool in the absence of GS-mediated assimilation.)

1.3.3.2.2 Symbiotic nitrogen fixers: The rhizobia

The rhizobia differ from the above nitrogen fixers in that they can fix nitrogen in symbiosis with plants. In this situation, ammonium is exported out of the bacteroids to the host plant cells. Two questions are of importance here: Does active ammonium transport function in an outward direction in this situation, and does uptake occur or is it repressed during symbiosis?

In 1982 Weigel and Kleiner published a survey of methylammonium uptake in several aerobic N_2-fixing bacteria, including *Rhizobium meliloti*. All strains showed uptake, and this was subject to inhibition by ammonium except in the case of *R. meliloti*. (The other strains were *A. vinelandii, Beijerinckia mobilis, Derxia gummosa, Azomas agilis, Azorhizophilus paspali, Azomonotrichon macrocystogenes, Xanthobacter autotrophicus* and *Alcaligenes latus*.) The level of uptake shown by *R. meliloti* was interpreted as due simply to unspecific diffusion across the cell membrane and immediate trapping

by assimilation. The authors suggested a general inability to synthesize ammonium transport systems in rhizobia, and an increased membrane permeability to ammonium: both adaptations to the symbiotic role of these organisms. However further work has shown this not to be the case, and ammonium/methylammonium carriers are in fact common in rhizobia.

Cowpea *Rhizobium* sp. strain 32H1 shows methylammonium uptake under certain conditions (Gober and Kashket 1983). This strain can fix nitrogen in the free-living state if the oxygen concentration is low, but not when it is high. Surprisingly, the O_2 concentration also seemed to regulate uptake activity, giving uptake for growth on 0.2% O_2 but not for growth on 21% O_2, despite the same low concentration of fixed nitrogen (6 mM glutamate) being supplied in each instance. These results suggest that nitrogenase and ammonium transport share a common regulatory system, but do not explain why uptake should be lacking under the very conditions where it would appear to be most necessary. The system was inhibited by ammonium (in a manner indicative of a shared carrier), and also by arsenate, azide, cyanide and other metabolic poisons - showing a requirement for metabolic energy. MSX also inhibited uptake, which was interpreted as evidence for the participation of GS in overall uptake, i.e. that total uptake consists of a combination of transport plus metabolism. However Marsh *et al.* (1984) reported uptake of both methylamine and ammonium to be higher in aerobically than in microaerobically-grown cells of the same organism (and also in bacteroids), but this has not since been confirmed.

Rhizobium leguminosarum MNF3841 can utilise methylammonium as a nitrogen source, and can be induced to exhibit a specific methylammonium permease distinct from the ammonium permease it can also synthesise (Glenn and Dilworth 1984). The methylammonium permease shows higher uptake of MA, and has a K_m for it of 0.035 mM. Ammonium does cause inhibition, but with only a K_i of 1.5 mM. By contrast, the ammonium

permease has a K_m for MA of 0.11 mM and a K_i for ammonium of 0.007 mM. Using a method involving an ion-specific ammonium electrode to measure ammonium-depletion of the medium rather than radiolabelled MA accumulation, the ammonium permease was further characterized (O'Hara *et al.* 1985). The K_m for ammonium was calculated as 0.015 mM, and the system was shown to be derepressed by nitrogen limitation (low ammonium, nitrate, glutamate or MA), in a manner inhibited by chloramphenicol. Glutamine was also inhibitory. Both permeases were shown to be sensitive to inhibitors of active systems.

Later work on *R. meliloti* itself did show ammonium uptake in this organism (Pargent and Kleiner 1985). Again ^{14}C-methylammonium uptake was used to characterize a carrier, which showed a K_m of 2 μM for MA and a K_i of 1.2 μM (competitive inhibition) for ammonium. Energy dependence was inferred by the ability to maintain a 100 fold concentration gradient of MA, and the inhibitory effect of metabolic poisons. Glutamine and asparagine also repressed the transporter.

Bradyrhizobium (Parasponia) sp. ANU289 also showed active uptake of methylammonium that was competitively inhibited by ammonium (Howitt *et al.* 1986). A K_m value of 6.6 μM was calculated for MA, with a K_i of 0.4 μM for ammonium. MSX was shown to cause complete inhibition of GS whilst only inhibiting uptake by 30%, whereas metabolic inhibitors such as azide caused 90% inhibition, indicating the contribution of metabolism to overall uptake. Again, derepression was subject to the nitrogen source and inhibited by chloramphenicol. A mutant showing less than 10% of the wild type uptake was isolated following Tn5 mutagenesis (Udvardi *et al.* 1992). However several other functions were also affected, and analysis showed the insertion to be in the regulatory gene *ntrC* (*glnG*). This indicates involvement of the Ntr system in the control of ammonium/methylammonium uptake in this organism.

Bradyrhizobium japonicum is an organism that can fix nitrogen in its free-living state as well as in symbiosis, in soybean root nodules. Udvardi and Day (1990) found that it possessed a methylammonium uptake system in free-living cells that were grown under nitrogen-limited conditions (K_m for MA: 2 μM). Unlike *R. leguminosarum*, *B. japonicum* cannot use MA as a sole nitrogen source, so the carrier was unlikely to be intended for MA. Ammonium was also shown to inhibit the carrier's synthesis and activity, and could cause efflux of accumulated label, indicating a preference for NH_4^+ over MA.

1.3.3.2.3 Ammonium transport in the symbiotic state

Laane *et al.* (1980) found that bacteroids of *R. leguminosarum* did not accumulate ammonium, but excreted it in response to the pH gradient. O'Hara *et al.* (1985) also investigated ammonium uptake by bacteroids of *R. leguminosarum* MNF3841 (using an ammonium electrode). The methodology used allowed isolation of bacteroids able to actively accumulate succinate, but no ammonium permease was found. Bacteroids of *Bradyrhizobium* sp. ANU289 were also assayed by Howitt *et al.* (1986) for methylammonium uptake. The rate of uptake by bacteroids was much less than for free-living cells, and was concentration-dependent and pH-dependent in a manner suggesting simple diffusion. Neither was it subject to inhibition by ammonium. *B. japonicum* bacteroids were shown to have active uptake systems for succinate and malate (Reibach and Streeter 1984) and were investigated by Udvardi and Day (1990) for methylammonium uptake, both across the bacteroid and the peribacteroid membrane. Uptake was similar for bacteroids and peribacteroid units (i.e. bacteroids still enclosed in the peribacteroid membrane, a membrane of plant origin that surrounds each individual bacteroid in the plant nodule), and was considerably less than for free-living cells. The rate was unaffected by uncouplers and ammonium, and appeared to be due to diffusion only, occurring down the

concentration gradient. Marsh *et al.* (1984) reported uptake of ammonium and ammonium-inhibited uptake of methylammonium in bacteroids of *Rhizobium* sp. 32H1, but this was not subsequently confirmed. Therefore it seems very unlikely that active ammonium uptake occurs in the bacteroids of symbiotic nitrogen fixing rhizobia. Export of ammonia to the plant is probably simply by diffusion. This raises the interesting question of how nitrogenase and the ammonium permease are regulated separately in this instance where they appear to have many regulatory features in common in other circumstances.

1.3.3.3 Cyanobacteria

Many cyanobacteria can fix atmospheric nitrogen, but will preferentially use fixed nitrogen sources, and have been often shown to use ammonia first if it is available (Boussiba and Gibson 1991). Therefore it is not suprising that there is evidence for the existance of ammonium permeases in these organisms.

The free-living cyanobacterium *Anabaena variabilis* showed uptake of methylammonium that was energy-dependent and capable of maintaining a 40 fold concentration gradient across the cell membrane (Rai *et al.* 1984). Ammonium was inhibiting and could cause efflux of label from cells. The pattern of uptake was biphasic in nature, with the second slower phase being susceptible to inhibition by MSX. The authors suggested therefore that overall uptake is a combination of transport and metabolism (via GS) and that only the latter is subject to MSX inhibition. They also investigated uptake by the symbiotic form of *Anabaena azollae*, and found a comparable system, differing only in a much slower second phase. This they attribute to the lower levels of GS in symbiotic cyanobacteria. Reglinski *et al.* (1989) isolated a methylammonium transport mutant of *A. variabilis*, with a reduced first phase and slower second phase. They characterized it as lacking NH_4^+/MA uptake but possessing a specific MA uptake system. Also, by using

mutants with reduced GS activity, they showed the second phase of uptake to be dependent on GS activity.

Anabaena dolıolum also shows the same bıphasıc pattern of methylammonium uptake, but both phases (and GS) can be ınactıvated by pretreatment for 3 hours wıth MSX (Sıngh *et al.* 1986). However an MSX-resıstant mutant (less effıcıent in transport of MSX) was only affected ın the second phase, and also retaıned ıts GS actıvity. The authors of thıs study ınterpret theır results ın terms of two dıfferent MA uptake systems, one of whıch ıs more sensıtıve to MSX, and dıstınct from GS actıvity.
There ıs also evıdence of ammonıum uptake ın *Anabaena flosaquae* (Turpın *et al.* 1984).

Boussıba *et al.* (1984) reported that several cyanobacterıa can maıntaın a faırly constant ınternal pool of ammonıum, ındependently of the nıtrogen growth condıtıons. They also found that *Anacystıs (Synechococcus) nıdulans* (whıch ıs unıcellular and non-nıtrogen-fıxıng) removed ammonıum from solutıon in a energy-dependent manner, wıth a K_m of less than 1 μM. MSX acted as an ınhıbitor, but methylammonium dıd not, and neıther was the uptake dependent on the nıtrogen source for growth. Uptake was pH-ınsensıtıve, unlıke fındıngs for other organısms. Thıs organısm ıs also capable of MA uptake (but at a lesser rate) whıch is subject to repressıon by ammonıum and has a requırement for Na^+ (Boussıba and Gıbson 1991). Kashyap and Singh (1985) reported two ammonıum uptake systems ın *A. nıdulans* (straın IU 625). They ıdentıfıed a low and a hıgh affınity system (K_m's of 357 μM and 50 μM respectıvely), that were both ınhıbıted by MA and MSX. Repressıon by growth on ammonıum was also reported, but at dıfferent levels for the two systems. They also showed energy dependence, and concluded that ATP was more ımportant for the low affınity system, but hıgh affınity uptake was more dependent on the proton gradıent.

High affinity (K_m 11 μM) and low affinity (K_m 66 μM) ammonium uptake systems have also been described in *Nostoc muscorum* (Kashyap and Johar 1984). Mutants lacking the low affinity system were isolated, one of which showed a link to reduced GS activity.

Nostoc sp. ANTH (which lives in symbiosis with *Antheroceros punctatus*) can, in free-living growth, utilise methylammonium as a sole nitrogen source (Prakasham and Rai 1991). When grown on N_2, it shows a biphasic pattern of methylammonium uptake, similar to that for other cyanobacteria, that is absent when grown on ammonium. However growth in methylammonium results in a pattern of uptake that is less obviously biphasic, and also higher. More significantly, the effect of added ammonium on uptake in this case is much less, not immediate, and does not cause efflux of label. This suggests a specific methylammonium system, as was previously found for *R. leguminosarum*. The specific methylammonium system was also insensitive to inhibition by MSX. Uptake by N_2-grown cells was affected by MSX (which, as for other cyanobacteria, inhibited GS and the second phase of uptake), even when assayed before enough time had elapsed for inactivation of GS to have occurred. Efflux of label is also reported to be caused by addition of MSX. This points to MSX having a direct inhibitory effect on the transporter as well as an effect on GS.

Nostoc calcicola is an alkalophilic nitrogen-fixer. Prasad and Kashyap (1990) showed that it can synthesise high and low affinity ammonium transport systems (K_m's of 10 μM and 200 μM respectively). However the pH ranges of these systems are approximately pH 6 - 8, whereas the natural environment of the organism is normally pH 10 - 11. This suggests active transport may not be necessary at high pH, as suggested by Boussiba and Gibson (1991) for two other alkalophillic cyanobacteria, *Spirulina platensis* and *Cyanospira rippkae*.

Kashyap *et al.* (1995) examined ammonium uptake by *Plectonema boryanum*, a filamentous non-heterocystous cyanobacterium that fixes nitrogen under microaerobic conditions of nitrogen stress. When grown on nitrate, a single ammonium uptake system appears to exist, with a K_m of 20 μM. It is not competitively inhibited by methylammonium, a fact attributed to the synthesis of specific methylammonium carriers. Nitrogen stress was shown to increase ammonium transport activity, and kinetic studies showed this to be due to the presence of two separate transport systems. A new low affinity system (with a K_m of 50 μM) existed in addition to a high affinity system identical to that already observed for growth on nitrate.

1.3.4 Conclusion

1.3.4.1 Overview

In summary, active systems for ammonium and methylammonium uptake appear to be very widespread in many different organisms. MA has certainly proved useful in elucidating ammonium transport, but by no means are the two types of transport one and the same. There is evidence of specific inducible methylammonium systems (Glenn and Dilworth 1984, Prakasham and Rai 1991) that are less susceptible to inhibition by ammonium than other MA-accepting carriers. More significantly, there is also evidence for the existence of ammonium carriers that do not accept the analogue (Boussiba *et al.* 1984, Cordts and Gibson 1987, Kashyap *et al.* 1995), indicating that the lack of methylammonium uptake in an organism does not preclude ammonium uptake by a carrier of narrower specificity. Multiple systems can exist in the same strain under certain conditions, (e.g. the high and low affinity systems described in cyanobacteria, [Kashyap *et al.* 1995], and in *R. capsulata*, [Genthner and Wall 1985]), a fact previously noted in eukaryotes (Wiame *et al.* 1985, Marini *et al.* 1994). The biphasic pattern of methylammonium uptake observed for most cyanobacteria and also for *A. vinelandii* (Gordon and Moore 1981) and *R. sphaeroides* (Cordts and Gibson 1987) has been variously attributed to two systems or to the respective contributions of transport first, followed by subsequent assimilation of the transported substrate. The glutamine analogue MSX inhibits many ammonium / methylammonium uptake systems, and must be transported into the cell to do so (Kleiner *et al.* 1983), but it is still unclear whether this is due to direct inhibition of a carrier or to the inactivation of GS. There is good evidence for the former view (Singh *et al.* 1986, Prakasham and Rai 1991), but it is nevertheless apparent that assimilation is an important component of overall uptake, even if GS itself is not directly responsible. Boussiba and Gibson (1991) suggest that amidation (i.e. by

GS) is critical in the regulation of nitrate and dinitrogen utilization in cyanobacteria, (by glutamine or a closely related compound) and probably also of ammonium / methylammonium uptake. This ties in with the inhibitory effect of glutamine found in many instances and also echoes the conclusions of Jayakumar and Barnes (1984) in their work on *A. vinelandii*. However, derepression of MA uptake in the latter organism was not sensitive to chloramphenicol, unlike in several others (e.g. *E.coli*, *R. leguminosarum*), indicative of regulation at a genetic level in at least some species. The Ntr system regulates ammonium uptake in *Bradyrhizobium (Parasponia)* sp. ANU289, as shown by the requirement for NtrC (Udvardi *et al.* 1992). There is strong evidence also for its involvement in the *E. coli* (Servín-González and Bastarrachea 1984, Jayakumar *et al.* 1986) and the *K. pneumoniae* (Kleiner 1982) systems. Ntr control is also suggested for other strains by the apparent "nitrogen control" effect of growth conditions.

1.3.4.2 Cyclic retention

The obvious function of an active ammonium carrier is to import ammonium into the cell when present in the environment in low concentrations. However Kleiner suggests a broader role for such a system as one of "cyclic retention" (Kleiner 1985). He compares the repression by its substrate (a rarity among transporters) with the similar repression of the high-affinity transport system for branched-chain amino acids in *E. coli*. The latter repression is accompanied by derepression of a low-affinity system, and together the two systems function to maintain a relatively constant intracellular pool of amino acids, despite wide variations in extracellular levels. (This appears to have some similarities with the two ammonium uptake systems described in *R. sphaeroides*, one of which is repressed by ammonium, which together maintain the intracellular ammonium level relatively constant [Cordts

and Gibson 1987].) The necessity for a system is explained by the loss of ammonia through outward diffusion when a higher concentration pertains inside the cell, e.g. when the organism fixes N_2, reduces nitrate or degrades organic nitrogenous compounds. A futile cycle of ammonia loss through diffusion and ammonium uptake by an active carrier is therefore invisaged to operate, the only exceptions being in the case of a high-ammonia environment and/or alkalophillic organisms (Kleiner 1985).

1.4 THE NITROGEN REGULATED (Ntr) SYSTEM

1.4.1 Nitrogen control

Investigations into the biochemistry of *E. coli* showed that certain enzymes and permeases involved in nitrogen assimilation were present at higher concentration if the cells were grown in nitrogen-deficient medium. Their synthesis was shown to be "nitrogen controlled" and regulated by a common system, which involved the *glnALG* operon. Genes under this control included *glnA* itself (the structural gene for glutamine synthetase), the *hut* genes (histidine utilization), *put* (proline utilization) and the genes for nitrate reduction (Magasanik 1982). In the nitrogen-fixing enteric bacterium *Klebsiella pneumoniae*, nitrogen fixation was found to be under the same control, designated the Nitrogen Regulated (or "regulatory") System. Since then, other genes not related to nitrogen metabolism have been found to be subject to the same control, the only common denominator in function appearing to be that all Ntr genes are not absolutely necessary for growth, but only under certain conditions (Merrick 1993).

1.4.2 Sigma factors

As mentioned previously with reference to regulation of *glnA* in *E. coli*, the Ntr system involves a specific sigma factor, σ^{54} (also called σ^{N}). A sigma factor is a protein that binds to core RNA polymerase (E; possessing the catalytic activity) to form the RNA polymerase holoenzyme ($E\sigma$) and conferring specificity for the initiation of transcription at a particular promoter sequence (Helmann 1994). The major sigma factor in *E. coli* is a 70 kDa protein known as σ^{70} (also called σ^{D}) that can promote transcription of all essential genes. There are also alternative sigma factors involved in the synthesis of groups of genes related to specialised functions (often in addition to transcription by σ^{70}), but most of these are

structurally related to the major factor and initiate transcription in the same manner. However σ^{54}, although it binds to the same core RNA polymerase, is distinctly different in both its structure and its mode of action, and is seen now as another separate family of sigma factors (Morett and Segovia 1993, Merrick 1993, Kustu *et al.* 1989, Helmann 1994).

1.4.3 Sigma 70

The sigma 70 family of sigma factors contains three main groups of proteins, showing overall significant homology and three to four highly conserved regions (Helmann 1994). Group I contains the major sigma factors that facilitate transcription of the majority of bacterial genes. Group II are a group of alternative sigma factors that show high sequence homology to Group I (e.g. the product of *rpoS*, specific to stationary phase genes in *E. coli*). Group III are alternative factors showing less homology to the major factors (e.g. sporulation-specific factors from *Bacillus* species, heat shock sigma factors). Transcription by RNA polymerase involves the steps of promoter recognition and binding, followed by DNA melting (open complex formation) and initiation of RNA manufacture. The most conserved regions in the sigma 70 family (designated 2 and 4) have been linked to binding to the core enzyme, DNA melting, and contact with the promoter sequence (Helmann 1994). The promoters recognised by this family of sigma factors show different consensus sequences, but always in the -35 and -10 regions relative to the start point for transcription. For σ^{70} itself (if not for the other members of its family), DNA recognition and melting appear to be strongly coupled, and ATP does not seem to be required for the melting process (Gralla 1993).

1.4.4 Sigma 54 (Sigma N)

Sigma 54 shows no sequence homology to the sigma 70 family of sigma factors, but has been clearly recognized as a sigma factor by its ability to bind to core RNA polymerase and initiate transcription at specific promoter sites (Kustu *et al*. 1989). Simga 54 homologues (varying in size from 47 - 59 kDa) have been found in both Gram-positive and Gram-negative bacteria, and have been implicated in the transcription of a wide variety of genes; for example - nitrate utilization, dicarboxylate transport, toluene breakdown, flagellin synthesis, nickle uptake and nitrogen fixation (Reviewed in Kustu *et al*. 1989, Merrick 1993, Morett and Segovia 1993, Atkinson and Ninfa 1994).

In contrast to σ^{70} and related sigma factors, the members of the σ^{54} family recognise a promoter in the -24/-10 region, and the actual sequence is highly conserved between the σ^{54}s of different species, more so than for any one σ^{70}-type sigma factor (Merrick 1993). Several workers have attempted to define this consensus, since a conserved promoter sequence in the -15 region (TTTTGCA) was first described for several *E. coli* and *K. pneumoniae* Ntr-dependent genes (Ow *et al*. 1983).

C T **G G** Y A R N4 T T **G C** A	(Johnston and Downie 1984)
C T **G G** Y A Y R N4 T T **G C** A	(Hunt and Magasanik 1985)
T T **G G** C A C A N4 T C **G C** T	(Reitzer and Magasanik 1986)
T **G G** C A C N5 T T **G C** W	(Morett and Buck 1989)

Table 1.1[2]: Consensus promoter sequences for σ^{54} suggested by various authors.

In all cases the strongly conserved GG and GC doublets appear to be an important motif in promoter recognition,

[2]Where Y = C or T, R = A or G, W = A or T.

and indeed have been shown to be by point mutation studies (Stigter *et al*.1993). Also the T residues preceding the GC doublet appear to have a role in binding stability (Buck and Cannon 1992).

Unlike σ^{70}, σ^{54} can bind to the promoter DNA in the absence of the core RNA polymerase, although its position appears to change by 3 - 4 bp in the presence of E (Buck and Cannon 1992). The core polymerase appears to enhance binding of σ^{54}, possibly by causing a conformational change (Cannon *et al*. 1993). However, the specificity and promoter recognition definitely reside in the sigma subunit. Another very important difference between σ^{70} and σ^{54} is that whereas $E\sigma^{70}$ is sufficient on its own to initiate transcription from a suitable promoter, $E\sigma^{54}$ also requires one of a group of specialised activator proteins. (A low level of transcription could be achieved from supercoiled *glnA* template in the absence of activator (NtrC), but this was increased 1000 fold by its presence; Hunt and Magasanik 1985). Also energy is required for $E\sigma^{54}$ transcription, in the form of ATP (Popham *et al*. 1989).

1.4.4.1 The *rpoN* gene.

The gene encoding σ^{54} was originally designated *glnF* (and later *ntrA*) and recognised as being involved in the expression of Ntr regulated genes in *E. coli* and *K. pneumoniae* (Magasanik 1982). Hunt and Magasanik (1985) identified its product as being the sigma factor of an RNA polymerase (binding to promoter DNA and core RNA polymerase) and therefore suggested the new name of *rpoN*. Since then, homologous genes have been discovered in various other species (Merrick 1993), including two copies of *rpoN*-like genes in *Bradyrhizobium japonicum* (Kullik *et al*. 1991). The expression of *rpoN* is normally constitutive (Merrick 1993).

1.4.4.2 Structure of the σ^{54} protein

The product of *rpoN* contains three distinct and highly conserved regions (Merrick 1993, Atkinson and Ninfa 1994, Helmann 1994, Wong *et al*. 1994). The amino-terminal portion of the protein (Region I) is rich in leucine and glutamine residues, and has a possible α-helical structure. The glutamines have been proposed to play a role in mediating the enhancer-dependent activation of transcription (Hsieh *et al*. 1994), and certain heptad leucine repeats appear critical for recognition of the -12 element of the promoter sequence and for mRNA production (Hsieh and Gralla 1994). The central section of the protein (Region II) is the least conserved among species, varying most in sequence length. It is notable for many acidic residues and also overlapping hydrophobic repeats, and appears (from mutational analysis) to be essential for binding of the core enzyme (Gralla 1993). The carboxy-terminal domain (Region III) is very highly conserved: a sequence of ten amino acids called the "RpoN box" are almost totally conserved from strain to strain. Another notable motif is a potential helix-turn-helix sequence, probably involved in DNA recognition. This region has been shown to be essential for binding to the DNA and for recognition of the -24 element of the promoter (Wong *et al*. 1994).

1.4.4.3 Activator proteins

As mentioned above, σ^{54} acts in conjunction with specialised activator or enhancer-binding proteins such as NtrC, another component of the Ntr system. Several other activator proteins have also been identified, and been shown to belong to a related family of proteins (Morett and Segovia 1993). Activator proteins have the ability to bind DNA at sites specific to each activator. These sites usually occur in pairs, separated by three turns of the helix, and are normally 100 to 200 bp upstream of the σ^{54} binding site. However they can still influence

transcription if removed much further away (e.g. 5 kb - NtrC activation of *E. coli glnA*; Reitzer and Magasanik 1986). This binding appears to be in order to increase the local concentration of the activator, whilst also of course having an element of specificity. In the case of NtrC at least, the occurrence of two adjacent binding sites is also important in allowing oligomerization of the NtrC dimers into a larger complex, necessary for transcriptional activation to take place (Porter *et al.* 1993). Binding of the activator can also serve to inhibit transcription from other promoters (e.g. by σ^{70}, Hirschman *et al.* 1985).

Sequence analysis of known activator proteins has shown them to have many features in common (Morett and Segovia 1993). They consist of three domains, between which the functions of the protein are quite strictly compartmentalised. The central domain is very highly conserved among different σ^{54} transcriptional activators, and appears to function in ATP binding / ATPase activity and interaction with $E\sigma^{54}$. There is also a small conserved region in the carboxy-terminal ends of the proteins, which appears to be a helix-turn-helix DNA-binding motif. The amino-terminal regions of these proteins show great variation in length and sequence. However a subgroup of these activators that belong to a family of two-component sensor-activator regulatory proteins (e.g. NtrC; its associated sensor being NtrB), do show homologies, believed to be related to a common method of activation, i.e. by phosphorylation.

1.4.4.4 Transcription by $E\sigma^{54}$

As stated previously, σ^{54} appears to be expressed constitutively, and can bind to its specific promoter sequence in the absence both of core RNA polymerase and of the associated activator protein specific to that particular gene. The binding of E will increase the stability of the bound complex, but it has been shown to

remain (in the absence of activator) a closed complex, i.e. the strands of DNA remain unmelted. The crucial step therefore in the initiation of transcription is the interaction of the upstream activator with $E\sigma^{54}$. In the case of NtrC, the activator must be oligomerised first (probably to a tetramer [Atkinson and Ninfa 1994]), a reaction favoured when the molecules have been phosphorylated (activated) by NtrB (Porter *et al* 1993). The interaction between the activator and $E\sigma^{54}$ has been shown to involve looping of the intervening DNA (Su *et al*. 1990).

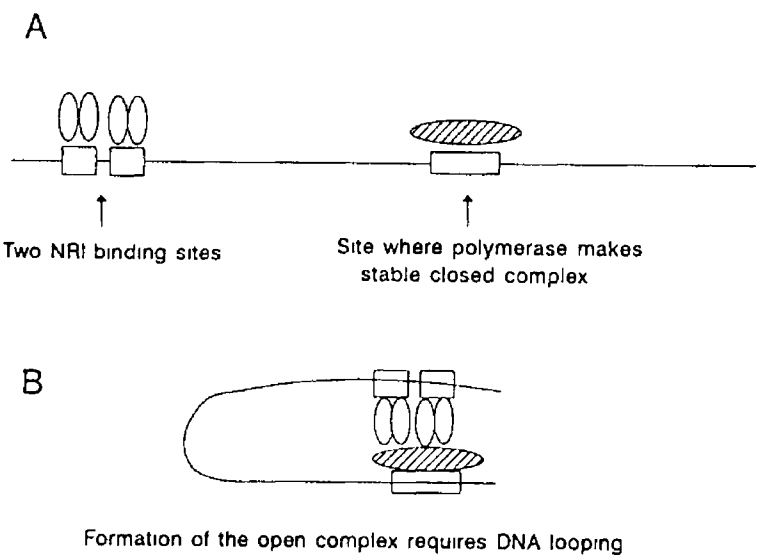


Figure 1.2 : Interaction between $E\sigma^{54}$ and activator protein for open complex formation. (Taken from Atkinson and Ninfa 1994.)

There is evidence that in some cases at least, the integration host factor (IHF; a heterodimeric protein known to cause bending of DNA by binding to AT-rich regions) assists by binding also to a site between the activator and $E\sigma^{54}$ (Santero *et al*. 1989, Cebolla *et al*. 1994). The

interaction of activator and $E\sigma^{54}$, and the hydrolysis of a molecule of ATP, results in the formation of an open complex, in which the DNA strands are melted. Transcription can then proceed without further involvement of the upstream activator protein. There is also evidence of the activator being able to effect transcription without binding to its usual upstream sites, providing it is in a high enough concentration (Reitzer and Magasanik 1986, Labes *et al*. 1993). This reinforces the idea that binding serves to increase the local concentration of the activator and hence its rate of interaction with the enzyme complex, rather than mediate an effect through contact with its actual binding site.

1.4.4.5 Comparison with eukaryotic transcription

Despite using the same core RNA polymerase, the differences between the modes of transcription of σ^{70} and σ^{54} are quite striking, particularly in the requirement shown by σ^{54} for an upstream activator, and in the uncoupled nature of the recognition and melting processes in which it is involved. In fact the σ^{54} system shows similarities to eukaryotic transcriptional processes in these features, for example to mammalian RNA polymerase II (pol II; Gralla 1993). Whilst the mammalian system requires more proteins than the three in the bacterial system (i.e. E, σ^{54} and the activator), there is a distinct division between them of the functions of recognition, activation, melting and polymerase binding, as between the different protein domains in the σ^{54} system. DNA binding and melting are definitely uncoupled, ATP is required, and distant upstream activators are also a feature. Some of the protein motifs in σ^{54} are also reminiscent of those in proteins of the pol II system, for example the glutamines and the leucine repeats found in its amino terminal region. So whilst σ^{54} may be something of an exception in bacterial transcription, it does have analogies elsewhere.

1.4.5 Biochemical basis of Ntr control

The Nitrogen Regulatory System is a transcriptional control system involving σ^{54}-RNA polymerase and its associated activator protein NtrC. Much of the elucidation of its action has come from study of the *glnALG* (or *glnA-ntrBC*) operon in *E. coli*. As mentioned previously, this codes for the only glutamine synthetase of *E. coli*, and for the NtrB and NtrC regulatory proteins. Regulation of this operon is quite complex, as it possesses three promoters, *glnAp1*, *glnAp2*, and *glnLp* (Hirschman *et al.* 1985, Reitzer and Magasanik 1986). It also possesses five NtrC binding sites upstream from the *glnA* start (See Figure 1.3). The *glnAp1* promoter appears to be a weak σ^{70} site, and transcription is stimulated by the catabolite activator protein (CAP) charged with cAMP. This can therefore maintain a low level of the three products in conditions of carbon deficiency and nitrogen excess. The *glnLp* (or *ntrBp*) promoter is also weak and σ^{70}-dependent, maintaining low levels of NtrB and NtrC only. However the *glnAp2* promoter is σ^{54}-dependent, also needing NtrC, and is transcribed under conditions of nitrogen deficiency to give high levels of the three gene products. The strongest NtrC-binding sites are those farthest from the start of transcription, and NtrC bound here is believed to interact with $E\sigma^{54}$ for transcription from *glnAp2*. The nearer sites may be involved in repression of the *glnAp1* promoter. NtrC appears to bind first to the stronger sites, even when in low concentrations and unable to activate transcription (presumably because it has not been activated by NtrB). Interestingly, lower concentrations of NtrC are able to activate *glnAp2* than are needed to activate other NtrC-dependent operons; a fact which makes sense given the products of *glnL* and *glnG*.

NtrB and NtrC have been recognised as being a pair of two-component sensor-activator regulatory proteins. Activation of NtrC requires phosphorylation of an aspartate residue in its N-terminal domain, hypothesised to allow the formation of tetramers and the ATPase activity necessary for it to

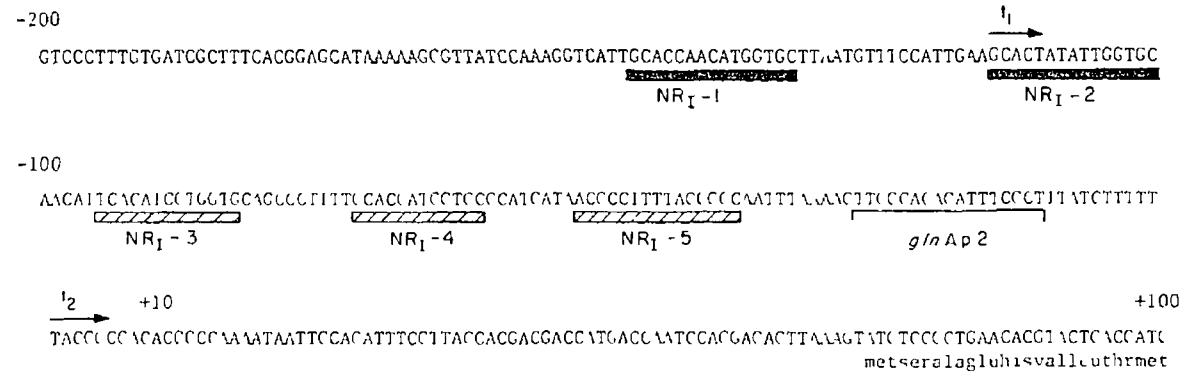


Figure 1.3 : The *glnA* promoter region of *E. coli*. The start sites of transcription from *glnAp1* and *glnAp2* are designated t_1 and t_2 respectively. The strong NtrC binding sites are shown by solid bars, and the weak sites by shaded bars. (Taken from Reitzer and Magasanik 1986.)

activate $E\sigma^{54}$ transcription (Atkinson and Ninfa 1994). NtrC has a limited ability to autophosphorylate from molecules such as acetyl phosphate (Feng *et al.* 1992), but the major control on its phosphorylation has been shown to be via NtrB, which has distinct kinase and phosphatase abilities (Atkinson and Ninfa 1993). NtrB autophosphorylates in low glutamine : α-ketoglutarate conditions, and then transfers the phosphoryl group to NtrC, but it is not itself the actual sensor of the nitrogen status of the cell. Two other proteins are involved - both mentioned previously with regard to adenylylation of GS - the P_{II} regulator protein and UTase (products of *glnB* and *glnD* respectively). UTase is also referred to as UT/UR because of its ability to catalyse both the uridylyltransferase and uridylyl-removing reactions interconverting P_{II} and P_{II}-UMP. It is now believed that the nitrogen-control response originates with this enzyme, which responds to nitrogen deficiency by uridylylating P_{II} to P_{II}-UMP This then stimulates the phosphotransferase activity of NtrB, possibly by a conformational change (Atkinson and Ninfa 1993, Kennedy *et al.* 1994), while also stimulating deadenylylation of GS by ATase.

1.4.6 Ntr in Rhizobia

Genes homologous to *rpoN, ntrB* and *ntrC* have been found in various members of the Rhizobiaciae (e.g. Szeto *et al.* 1987, Ronson *et al.* 1987, Udvardi *et al.* 1992, Patriarca *et al.* 1993). However the organisation of these genes is different from that in *E. coli* and the enterics. In *R. etli*, the *ntrBC* genes are part of an operon containing an unknown upstream ORF that is not *glnA;* their transcription is not σ^{54}-dependent, does not increase for growth on nitrate, and appears to be negatively autoregulated (Patriarca *et al.* 1993). In *R. leguminosarum*, *glnB* is co-transcribed with *glnA*, and regulated by NtrC and NtrB (Chiurazzi and Iaccarino 1990). In addition to the role of its product in NtrB/NtrC activation, P_{II} also seems to have a separate role in regulation of the genes for nitrate utilization (Amar *et al.* 1994).

Mutants in *ntrC* have also been shown to display phenotypes different to the mutant phenotype in enteric bacteria. In addition to the ammonium assimilation differences due to there being more than one GS in this family of organisms, *ntrC* mutants are not amino acids auxotrophs. There is also variability between strains in the ability of *ntrC* mutants to use nitrate as the sole nitrogen source (Szeto *et al.* 1987, Udvardi *et al.* 1992, Moreno *et al.* 1992).

1.4.6.1 Control of nitrogen fixation

In *K. pneumoniae*, the expression of the nitrogen fixation (*nif*) genes is tightly regulated. Firstly, fixation is an energetically expensive process, so is only undertaken as a last resort when there is no fixed nitrogen source available in the environment. Secondly, nitrogenase is extremely sensitive to oxygen, therefore it would be wasteful to produce it in high O_2 conditions. As a consequence, this organism has two systems of control operating with regard to *nif* expression, namely the

nitrogen-sensitive Ntr system and the *nif*-specific oxygen-sensitive control mediated by the *nifLA* operon (Ausubel 1984, Gussin *et al.* 1986). This operon is transcribed by $E\sigma^{54}$ with NtrC as activator (and is therefore under Ntr control itself) and encodes a pair of sensor-activator regulatory proteins, NifL and NifA. These show some similarity to the NtrB/NtrC pair, except that NifL is a sensor of oxygen rather than nitrogen status. NifA is another enhancer-binding protein capable of activating σ^{54}-dependent transcription, in this case of the *nif* genes, and indeed can also substitute for NtrC in activating certain nitrogen-regulated operons, including its own (Drummond *et al.* 1983, Ow and Ausubel, 1983). Unlike NtrC, NifA appears to be synthesized in an active form, and is inactivated by NifL in conditions of high O_2 (Gussin *et al.* 1986).

In contrast to the situation in free-living diazotrophs, the control of symbiotic nitrogen fixation in the rhizobia does not appear to have the same strong element of nitrogen control - presumably because of their symbiotic relationship (Reviewed in Fischer 1994). Although (unlike for *K. pneumoniae*), NtrC can also activate transcription of some of the *nif* promoters in the rhizobia (Sundaresan *et al.* 1983b, Szeto *et al.* 1987), *ntrC* mutants have been shown to be unaffected symbiotically (Szeto *et al.* 1987, Udvardi *et al.* 1992). As before, σ^{54} and NifA are required for transcription of the other *nif* genes (homologous to those in *K. pneumoniae*) and also of *fixABCX* - genes also essential for symbiotic nitrogen fixation in rhizobia. There is also evidence of a direct inhibitory effect by O_2 on rhizobial NifA, in adddition to that mediated by NifL (Fischer *et al.* 1988, Morett *et al.* 1991).

There are also two other regulators important in the control of nitrogen fixation in rhizobia, namely FixJ and FixK - neither involving σ^{54}. The details of the regulatory circuits differ between the genera of the rhizobia, but each contains these elements (Fischer 1994). FixJ is a transcriptional activator, showing homology to NtrC, and is

co-transcribed with FixL from the *fixLJ* operon. The latter is a membrane-bound sensor protein sensitive to microaerobiosis (David *et al.* 1988). It contains a heme-binding oxygen-sensitive module and kinase activity, while FixJ has a regulatory domain (where it is probably phosphorylated) and an activator domain that shows some homology with sigma factors (Reyrat *et al.* 1993). However unlike the two-component sensor-activator systems described already, FixJ activates transcription by the major sigma factor, σ^{70}. In *Rhizobium* species, FixJ has a direct positive regulatory effect on *nifA*, and also on *fixK*. The latter gene encodes FixK, the activator controlling the remaining fixation genes, *fixNOQP* and *fixGHIS*. This protein shows homology to a family of transcriptional activators that includes the *E. coli* Fnr and Crp proteins (Colonna-Romano *et al.* 1990).

1.4.6.2 Additional genes required in symbiosis

The *nod* genes are another set of rhizobial genes (along with *nif* and *fix*) required for successful symbiotic nitrogen fixation. Their products are elicited in response to plant factors, and consist of substituted lipooligsacharide signal compounds, essential for the effective infection/nodulation of the plant by the microorganism. The induction of the *nod* genes (*nodD* and *nodYABC*) is subject to repression by ammonium, but in *B. japonicum*, this was reported to be independent of Ntr/Nif systems (Wang and Stacey 1990). However Dusha and Kondorosi (1993) have shown that in *R. meliloti*, mutations in *ntrC* and *rpoN* cause a delay in nodulation, and that elements of the Ntr system interact with the *syrM-nodD3* operon, where *nodD3* codes for an activator protein necessary for expression of the *nodABC* genes. They have shown NtrC binding sites upstream from *nodD3*, and also the repressive effect of another gene *ntrR* (in conditions of high nitrogen) via *syrM*. (A mutant in *ntrR* was also shown to allow higher expression of *glnII* under normally repressing conditions, therefore appearing to be another

component of the Ntr system rather than a nodulation-specific factor [Dusha *et al.* 1989]. However it does not appear to have been further characterised to date.)

Another system shown to be essential in symbiosis is the C_4-dicarboxylate carrier encoded by *dctA*, a transporter that imports succinate, malate, fumarate and aspartate into free-living cells of *R. meliloti* and *R. leguminosarum*, and also into bacteroids (Engelke *et al.* 1989). Expression of *dctA* is σ^{54}-mediated, and also dependent on the products of a related operon, *dctBD*, whose products are another pair of sensor-activator regulatory proteins. DctB is a membrane-bound sensor that activates DctC by phosphorylation (Giblin *et al.* 1995), the latter being an enhancer binding-protein with considerable homology to NtrC and NifA. It has been shown to bind upstream of the σ^{54} promoter in a similar fashion to NtrC (Ledebur *et al.* 1990), and to promote transcription when activated, but have a negative effect if in its unactivated form (Labes and Finan 1993). The *E. coli* CRP activator has been shown to have a negative effect on *dctA* expression by also binding to the DctD upstream sites (Wang *et al.* 1993). It is interesting that although *dctA* is an absolute requirement for symbiosis, *dctB* and *dctD* are not (Yarosh *et al.* 1989). This suggests that another activator may function to initiate transcription of *dctA* under symbiotic conditions, possibly NifA.

1.4.7 Conclusion

The alternative sigma factor σ^{54}, is a key component not only of the Ntr system as such, but also of several other related (and also of apparently unrelated) systems of transcriptional control in bacteria. In the rhizobia, it is essential for symbiosis because of its involvement in the control of nodulation (in *R. meliloti* at least) and also of the NifA-dependent nitrogen fixation genes, and the C_4-

dicarboxylate transport system. The activators promoting σ^{54} initiation of transcription (e.g. NtrC, NifA, DctD) appear to be the means by which specificity is conferred, but there is also a certain amount of "crosstalk" between them. This is shown by the fact that high concentrations of NtrC for example, can activate promotion at NifA sites - probably without binding to any upstream sequence (Labes *et al.* 1993) - and also in the fact that DctD and NtrC are not essential in fixation. A mutant in *dctB* has also been isolated that affects *nod* expression in *R. leguminosarum*, but whether this is a direct effect of DctD or a more general metabolic effect is unclear (Mavridou *et al.* 1995). However the use of σ^{54} rather than σ^{70} in the transcription of certain operons clearly enables the cell to exert a high level of control over the expression of specialised groups of genes not always necessary for its metabolism, hence allowing greater overall efficiency.

Chapter 2 :
Materials and Methods

2.1 MATERIALS

2.1.1 STRAINS

The following table lists the strains used in this work, their genotypes and sources.

E. coli		
DH5α	F⁻, *end*A1, *hsd*R17(r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*ZYA), U169, *deo*R, λ-, θ80d*lac*ZΔM15.	Hanahan (1983)
INVαF′	*end*A1, *rec*A1, *hsd*R17(r^{-k} m^{+k}), *sup*E44, λ⁻, *thi*-1, *gyr*A, *rel*A1, φ80*lac*ZαΔM15Δ(*lac*ZYA-*arg*F), deoR+, F′	Invitrogen, based on Hanahan (1983)
R. meliloti		
1021	Wild type SU47 Sm^r	Meade *et al.* (1982)
1680	*ntrA1*::Tn5 Sm^r Nm^r	Ronson *et al.* (1987)
5001	*ntrC*::Tn5 Sm^r Nm^r	Szeto *et al.* (1987)
5003	*ntrC*::Tn5 Sm^r Nm^r	Szeto *et al.* (1987)
5422	*ntrA75*::Tn5	Finan *et al.* (1988)
G649	*ntrC283* Ω 5199::Tn5-233	Labes *et al.* (1993)
1-20	*glnA*::Tn5 Sm^r Km^r	de Bruijn *et al.* (1989)
2-37	*glnII*::Tn5 Sm^r Km^r	de Bruijn *et al.* (1989)
3-5	*glnT*::Tn5 Sm^r Km^r	de Bruijn *et*
4-15	*glnA*(MudIIPR46)*glnII*::Tn5 Km^r Sm^r Gm^r	de Bruijn *et al.* (1989)

Table 2.1 : Strains used in this work.

2.1.2 MEDIA

M9 mannitol (A variation on M9, Sambrook *et al.* 1989.)

$Na_2HPO_4.2H_2O$	6.6 g
KH_2PO_4	3.0 g
mannitol	2.0 g
NaCl	0.5 g
H_2O	1 litre
pH adjusted to	7.4

The above was autoclaved separately before adding the following:

vitamins	5.0 ml
salts	3.0 ml
iron	2.0 ml
Nitrogen source	As required

Vitamins: 20 mg each biotin, pantothenic acid, thiamine, in 1 litre, filter sterilized.

Salts: The following were autoclaved separately, then pooled - 8.213g $MgSO_4.7H_2O$ in 25 ml
0.247g $CaCl_2.2H_2O$ in 25 ml

Iron: 0.004g $FeCl_3$ in 50 ml 10mM HCl (autoclaved).

Luria-Bertani medium (LB) (Millar 1972)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
H_2O	1 litre

SOC medium (Sambrook *et al.* 1989)

Tryptone	5.0 g
Yeast extract	5.0 g
NaCl	0.5 g

The above was dissolved in 950ml deionized H_2O, 10ml 250mM KCl added, the pH adjusted to 7.0 (using 5M NaOH) and the volume brought to 1 litre.

Following autoclaving, 5ml 2M $MgCl_2$ (autoclaved separately) and 20ml 1M glucose (sterile filtered) were added.

Tryptone-Yeast extract medium (TY) (Beringer 1974)

Tryptone	5.0 g
Yeast extract	3.0 g
$CaCl_2.2H_2O$	1.32g
H_2O	1 litre
pH	7.2

Yeast extract - Mannitol (YM) (Nuti *et al.* 1977)

K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.4 g
Mannitol	10.0 g
H_2O	1 litre
pH	6.9

Solid Media

To make solid media, Oxoid Technical Agar # 3, was added at 1.2% prior to autoclaving.

Antibiotics

ampicillin: 25 μg/ml in broth, 100 μg/ml in agar
kanamycin: 25 μg/ml in broth, 50 μg/ml in agar
neomycin: 30 μg/ml in broth, 60 μg/ml in agar
gentamicin: 50 μg/ml.

X-gal

5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) was used as a substrate for the detection of plasmids carrying inserts in the *lacZ* gene when grown in an appropriate host (White instead of blue colonies). A solution of 20 mg/ml X-gal in dimethylformamide was spread over each set agar plate to be used (40 μl / plate), and left to dry before spread-plating the culture.

2.1.3 BUFFERS AND SOLUTIONS

(Sambrook *et al.* 1989, unless otherwise stated)

Tris Acetate EDTA (TAE)

(Used for agarose gel electrophoresis)

A 50X stock contained:

242g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA, pH 8.0
in 1 litre H_2O.

This was autoclaved, then diluted with H_2O as required.

Tris Borate EDTA (TBE)

(Used for polyacrylamide gel electrophoresis of sequencing reactions)

A 10X stock contains:

108g Tris-HCL
9.3g Na_2-EDTA
55g Boric acid
in 1 litre H_2O.

The pH was adjusted to 8.3, and the solution autoclaved, then diluted as required.

Tris EDTA (TE)

(Routinely used for DNA solutions)

1.21g Tris-HCl
0.37g Na_2-EDTA
in 1 litre H_2O.

The pH was adjusted to 8.0, and the solution autoclaved.

Tris EDTA Sodium Chloride (TES)

1.21g Tris-HCl
0.37g Na_2-EDTA
2.92g NaCl
in 1 litre H_2O.

The pH was adjusted to 8.0, and the solution autoclaved.

STET solution

(For plasmid DNA minipreps)

8% glucose

0.5% Triton X 100

50mM EDTA

50mM Tris-HCl pH 8.0

Phenol / Chloroform (Kirby Mix)

(For general DNA extractions)

100g phenol

100ml chloroform

4ml isoamyl alcohol

0.8g 8-OH quinolone

[The phenol must be equilibrated first with 100 mM Tris pH 7.5 until the correct pH is obtained (assessed by taking the pH of the aqueous layer), then the other components added.]

Store under 100mM Tris-HCl pH 7.5 at 4°C.

Sodium Iodide solution

(Based on Vogelstein and Gillespie 1979)

Sodium iodide (90.8g) was stirred in 100ml water until as much as possible had dissolved, then 1.5g sodium sulphite was added. The solution was then stored at 4°C in a dark bottle.

Ethanol Wash (Based on Vogelstein and Gillespie 1979)

50% ethanol

10mM Tris-HCl pH 7.5

1mM EDTA

Stored at -20°C.

2.2 METHODS

2.2.1 Methylamine transport assay

The assay procedure used was based on that of Jayakumar and Barnes (1983).

Cells to be assayed were grown (generally for 4 days at 30°C with shaking) in minimal medium (M9-mannitol) plus the appropriate nitrogen source (e.g. 0.5mM NH_4Cl), then harvested by centrifugation at 10,000rpm for 10 mins (Beckman J2-21, JA-20 rotor), and washed twice in sterile water to remove all traces of the medium. They were then resuspended in the same basic medium, but without any nitrogen source, to a concentration based on the initial OD_{550nm} of the culture. (An initial 10ml sample of culture was given a resuspension volume in ml of O.D.x 2.5). The actual assay was carried out as follows: ^{14}C-methylamine (NEN Research Products #NEC-061) to give a concentration of 9.6μM, was mixed with the cell suspension at time zero, and 100μl samples then taken at timed intervals. (Total reaction volume used was generally 250 or 500μl). Each sample was immediately vacuum filtered through a Nucleopore® polycarbonate 0.45μm membrane (Costar) overlaid on damp Whatman#4 paper, to remove the surrounding medium. Each filter was then placed in a scintillation vial with 5mls scintillation fluid (Ecoscint H, National Diagnostics), and the carbon-14 counts per minute (cpm) determined with a Beckman LS 7500 Liquid Scintillation Counter (program #3, channel 2 values), thus giving a measure of the radiolabel taken inside the cells. As a control, 10 μl of unfiltered reaction mix was also taken and counted in each instance.

2.2.2 Bradford assay

This method was used to determine the total protein of cell suspensions assayed for methylamine uptake ability, and derived from that of Bradford (1976). The standards used were Bovine Serum Albumin (Sigma) at concentrations of 0.1 - 1.0 mg/ml, in water A water blank was also used (to zero

the spectrophotometer), but only the most linear region of the standard curve was used in the calculation of sample values, generally 0.1 - 0.6 mg/ml (due to the use of NaOH in the procedure). Samples and standards were treated as follows (all in duplicate) : 100μl of sample was added to an equal volume of 1M NaOH in a microfuge tube, and placed in a boiling waterbath for exactly 10 mins. Diluted Bradford reagent (Biorad; 800μl of a 1 in 5 dilution in deionised water) was added to each tube. The O.D. of each sample was read at 595nm (after zeroing with the water blank), and a standard curve drawn from the standard values (using Sigmaplot™, Jandel Scientific). Protein concentrations for the unknown samples were then computed from the graph, taking account of dilution factors, if necessary.

2.2.3 Boiling miniprep (STET method)

The following method was based on that of Holmes and Quigley (1981) and used for the isolation of plasmid DNA. An overnight culture (1.5 ml) was spun in a microfuge for 1 min. The supernatant was removed and the pellet resuspended in 0.35 ml STET solution. A fresh lysozyme solution (10 μl of a 10 mg in 333 μl of 10mM Tris pH 8.0 solution) was added. The tube was vortexed in one quick burst and placed in a boiling waterbath for exactly 3 min, then immediately spun in a microfuge for 15 min at RT. The supernatant was transferred to a microfuge tube containing 0.33 ml isopropanol, and vortexed. This was then frozen for at least 15 min at -70°C, before being spun for 10 min at 4°C. The supernatant was discarded, and the pellet dried under vacuum, before being resuspended in 100μl TE.

2.2.4 Qiagen column method

Plasmid DNA was also purified using the Qiagen Plasmid Midi Kit (Qiagen Inc., USA), in which an affinity resin column and the appropriate buffers are supplied. Purity and yield were higher than for the STET method. A single colony was

used to inoculate 5ml of LB (plus the appropriate antibiotic) which was grown overnight, then used to inoculate a further 150ml of the same medium. (This was more than enough for a high copy number plasmid, given the capacity of the column, as evidenced by the presence of plasmid in both the flow-through and the wash.) This was again grown overnight, and harvested by centrifugation in a Beckman J2-21 (JA-14 rotor) at 6,000 rpm for 15 min at 4°C. The pellet was resuspended in 4ml buffer P1, and transferred to a glass "Corex" tube. Buffer P2 (4 ml) was added, mixed, and the tube incubated at RT for 5 min. Then 4ml buffer P3 (chilled) was added, mixed gently, and further incubated, on ice, for 15 min. This was then spun (JA-20 rotor) at 16,000 rpm for 30 min at 4°C, but if the resulting supernatant was not fully clear, the step was repeated for a further 15 min, and the clear supernatant then promptly removed. A Qiagen-tip 100 column was equilibrated with 4ml buffer QBT just before use, then the supernatant applied and allowed to flow through. The column was then washed with 2 x 10ml buffer QC, and eluted with 5ml buffer QF. The eluent was collected into a "Corex" glass tube, 0.7 volumes of isopropanol added, and immediately centrifuged at 12,000 rpm and 4°C for 30 min. This served to precipitate the DNA, which was visible after as a faint feathery pellet. The pellet was then washed with 70% ethanol, spun again, and air dried, before being resuspended in 100μl TE, and stored at 4°C.

2.2.5 Isolation of total DNA

A 10ml TY culture was spun for 5 mins at 10,000 rpm. (Beckman J2-21 centrifuge, JA-20 rotor). The supernatant was removed, and the pellet washed with 5ml TES buffer, and spun down again. After again removing the supernatant, the pellet was resuspended in 5ml TE. Freshly prepared lysozyme (0.5 ml of a 2mg/ml solution in TE) was added, and the tube incubated at 30°C for 20 min. Then 0.5ml of a sarkosyl/pronase solution was added, followed by incubation
(10% sarkosyl and 5mg/ml pronase in TE)

at 37°C for 1 hour. Sodium acetate pH 5.2 (0.7ml of a 3M solution), and 2.5ml Kirby Mix were then added, and the tube mixed by inversion until an emulsion had formed. This was then spun at 10,000rpm for 10 min, and the resulting upper layer (aqueous) removed to a glass "Corex" tube, in which it was spun again for 15 min. The supernatant was then carefully removed, so as to take as much of the clear viscous material as possible, whilst leaving behind the white precipitate. This supernatant was then subjected to a second extraction with Kirby Mix, retaining the upper aqueous layer as before, which was further extracted again, this time with a 24:1 mixture of chloroform:isoamyl alcohol. The upper layer from the final extraction was then added to an equal volume of isopropanol, and mixed by inversion until a "thread" of DNA became visible in the mixture. This "thread" was removed from the tube with a micropipette, and placed in a microfuge tube, where it was spun for 5 mins to pellet the DNA. The supernatant was discarded, and the pellet washed with 70% ethanol and spun (twice). It was then dried under vacuum, and finally resuspended in TE (100-500μl).

2.2.6 Electrocompetent cells

A 10ml LB culture of *E. coli* DH5α was grown overnight at 30°C with shaking, then 5ml of it used to inoculate a fresh 500ml of LB. This was then grown in a shaking waterbath (37°C) and the optical density at 600nm was monitored until an OD of 0.5 - 0.8 was reached. The incubation was then stopped, and the flask chilled on ice for 15-30 mins, following which the cells were pelleted by spinning at 5000rpm in a Beckman J2-21 centrifuge (JA-14 rotor) for 15 min at 4°C. The supernatant was discarded and the cells resuspended in 500ml chilled sterile water by shaking gently on ice. The centrifugation was repeated, and the cells resuspended in the same way in 200ml chilled sterile water, then centrifuged again. The next resuspension was in 10ml ice cold 10% glycerol, followed by another spin. The

final resuspension was in 1.4 ml 10% glycerol, which was then split into 100 µl aliquots in sterile microfuge tubes, and stored at -80°C until required (expiry of 6 months).

2.2.7 Electroporation

A Biorad Gene Pulser was used, and 0.1 cm electroporation cuvettes. The electroporator settings were as follows: 1800 Volts, 200 Ohms, 25 µFD capacitance and 125 µFD capacitance extension. A volume of between 2 and 5 µl of DNA was used. TE was used as a negative control, and 0.2 ng pUC18 as a positive control, from which the electroporation efficiency (number of transformant colonies per µg DNA) was calculated. All transfers were performed asceptically, and as quickly as possible.

Competent cells were thawed at room temperature, then left on ice, along with the sterile cuvettes. The DNA solution was added to an aliquot of cells, which was left briefly on ice, before being transferred to a cuvette. The cuvette was wiped with tissue to remove moisture from the outside, tapped off the bench to dislodge any bubbles, then placed in the sliding holder of the electroporator and pushed into place. The pulse was applied by depressing the two red buttons simultaneously and holding until a "beep" sounded. The cuvette was then quickly removed and 1ml of LB added immediately and mixed by pipetting. The contents of the cuvette were then transferred to a microfuge tube and incubated at 37°C for 1 hour, then plated onto LB agar containing ampicillin (and X-Gal, if required). Dilutions of 10^0 and 10^{-1} were generally used (diluted in LB), and 100µl spread per plate. Plates were incubated at 37°C overnight, after which transformant colonies were visible, and blue and white morphologies were distinguishable (if X-Gal was being used).

2.2.8 Extraction of DNA from agarose gels

This method for the recovery of DNA fragments from agarose gels was based on Vogelstein and Gillespie (1979) and the Boehringer Mannheim Agarose Gel DNA Extraction Kit.
The appropriate sections of gel were cut out, weighed, and divided between microfuge tubes, to give no more than 0.5g per tube. Sodium Iodide Solution (2-3 vols) was added, and the tubes incubated at 55°C until the agarose had fully dissolved. A suspension of silica 325 mesh glass beads (2 - 5 μl) was added to each, and the tubes were mixed and left at room temperature for 5 min, before being spun in a microfuge for 5 seconds. The supernatants were discarded, leaving the glass beads, which were washed three times with 100μl Ethanol Wash, spinning for 5 seconds each time. The beads were finally resuspended in 10μl TE per tube and incubated for 3 mins at 55°C to elute the DNA. A final spin of 30 seconds was used to pellet the beads, after which the supernatant was removed and stored at 4°C.

2.2.9 Restriction

Restriction of DNA was generally carried out at 37°C for 1 hour, and in a total volume of 20μl, including 2μl of the appropriate 10x buffer, and 1μl of the enzyme. For large scale partial digests, this was scaled up to 35μl, and the enzyme was diluted (in the same buffer), and the time shortened in order to achieve the desired degree of restriction.

2.2.10 Ligation

Ligations (apart from those for cloning of PCR products) were performed in a total volume of 5μl, to achieve a good concentration of insert and vector DNA. The appropriate volumes (ie. to give desired DNA content) of both DNA solutions for the reaction were first mixed, and

precipitated by adding one tenth the volume of 3M sodium acetate pH 5.2, and two volumes ethanol, and leaving at -80°C for at least 15 mins. This was then centrifuged at 12,000 rpm in a microfuge, the supernatant removed, and the pellet dried. This pellet was subsequently resuspended for the ligation reaction in 3μl sterile water, 1μ 5x ligase buffer, and 1μl T4 DNA ligase. The reaction was then allowed to procede overnight at 14 - 18°C. Ligation efficiency was assesed by using the resulting solution to transform electrocompetant cells.

2.2.11 Agarose gel electrophoresis

Routine separation of DNA was achieved by electrophoresis at 100 volts through 0.8 % agarose (Molecular Biology Grade, Promega) in TAE, followed by staining in 1 μg/ml Ethidium Bromide to allow visualization of the bands on a UV transilluminator. For fragments smaller than 1 kb (e.g. small PCR products or restriction fragments), 2.0 - 4.0 % gels were used, and Nusieve agarose (FMC) for its better separation properties at that concentration.

2.2.12 Photography

Photographs were taken using an Olympus OM-20 camera and T-Max 100 black and white film (Kodak). An A003 red filter (Corkin, France) was used to block UV light when necessary, and also close-up lenses (Vivitar) were fitted when required. Negatives were developed using a 35 mm Film Tank (Photax), the reel of film being loaded onto the spool and placed in the tank in darkness. This system then allowed the developing and fixing steps to take place outside the darkroom without exposing the film. Kodax D76 developer and Unifix fixer were used as specified by the manufacturer. Prints were later made onto Kodak Kodabrome II RC F4 high contrast paper using an enlarger (darkroom), developed with Polymax (Kodak) and fixed also with Unifix fixer.

2.2.13 Polymerase Chain Reaction (PCR)

A Hybaid Omnigene Thermal Cycler, fitted with a 48 well heating block taking 0.5ml tubes, was used for all PCR reactions. Reaction volume was always 50 µl, plus 50 µl of sterile paraffin overlay. Reaction Buffer (10X), 25mM $MgCl_2$, dNTPs (100mM solutions of each) and *Taq* DNA Polymerase were all supplied by Promega. Reactions were set up aseptically, and all components kept on ice. Reaction products were analysed by running 5 µl samples on 2 % Nusieve agarose minigels alongside suitable markers.

2.2.14 Cloning of PCR products

PCR products were cloned using the TA Cloning® Kit (Invitrogen). Fresh PCR products were used, if possible immediately following verification by gel electrophoresis that amplification had occurred. This was in order to avoid loss of the 3'A-overhangs on the amplified fragments that are utilised in the cloning procedure. (The vector, pCR™II, contains 3' T-overhangs in the linear form in which it is supplied in the kit.) No purification needed to be carried out on the PCR products before ligation, and in all cases the maximum volume of PCR product compatible with the kit procedure was utilised. The ligation reactions therefore consisted of: 1 µl 10X ligation buffer, 2 µl resuspended pCR™II vector (25 ng/µl), 6 µl PCR product and 1 µl T4 DNA ligase. Incubation was at 14-15°C overnight, and transformations were carried out the following day using the kit protocol, with kanamycin as the selective antibiotic (as the PCR products in question had been amplified originally from an ampicillin-resistant template). This involved the following: adding 2 µl of 0.5M ß-mercaptoethanol (supplied) to each 50 µl vial of competent cells (*E. coli* INVαF') followed by 1 µl of ligation mix (with very gentle mixing each time), incubating on ice for 30 mins, at 42°C for 30 seconds, and again on ice for 2 mins, adding 450 µl prewarmed SOC

medium, and shaking at 37°C for 1 hour at 225rpm, before finally leaving on ice and plating onto LB agar containing antibiotic and X-gal.
White colonies were subsequently picked for plasmid isolation and analysis.

2.2.15 DNA Sequencing

The Sanger method of dideoxy-mediated chain termination was used, based on Sanger *et al.* (1977). Reagents for sequencing reactions were from the Pharmacia ^{T7}Sequencing™ Kit, and the protocols for annealing of template and the sequencing reactions were as described in the accompanying booklet.

2.2.15.1 Denaturation of template and annealing of primer.

The template DNA used in each case was a plasmid (pCR™II) containing a cloned PCR fagment, and was purified using the Qiagen column method. The primer used was either the Universal Primer (^{T7}Sequencing™ Kit) or Sp6 Promoter Primer (Promega). The method of annealing was Procedure C: Standard Annealing of Primer to Double-Stranded Template. The DNA was first denatured with NaOH as follows:
1.5 - 2 μg of DNA was made up to 24 μl with TE buffer in a microfuge tube, and to this was added 16 μl of 1M NaOH. This was mixed and centrifuged briefly, then incubated at room temperature for 10 min. The NaOH was then separated out by ethanol precipitation: 7 μl of 3M sodium acetate (pH 4.8), 4 μl of sterile deionised water and 120 μl of 95% ethanol were added, mixed, and the tube placed at -80°C for 15 min, before being centrifuged for 15 min to pellet the precipitated DNA. The pellet was washed once with ice-cold 70% ethanol and recentrifuged for 10 min, before being dried for 2 min in a Savant DNA Speed Vac®. Sterile water (10 μl) was used to resuspend the denatured DNA, which was used immediately in the annealing reaction. This entailed

adding 2-3 μl of the appropriate primer and 2 μl of annealing buffer to the DNA solution, mixing and centrifuging briefly, then incubating the reaction at 65°C for 5 min, 37°C for 10 min, and at room temperature for at least 5 min.

2.2.15.2 Sequencing reactions

While the annealing reaction was proceeding, the sequencing reaction tubes were prepared by adding 2.5 μl of the appropriate "read short" mix to a labelled microfuge tube (A, C, G and T). An appropriate amount of diluted T7 DNA Polymerase was also prepared by diluting 1:4 with enzyme dilution buffer (supplied with the kit), and kept (with the reaction mixes) on ice. On completion of the annealing reaction, the following were added to the tube containing the annealed primer/template: 3 μl labelling mix-dATP, 1 μl [α-^{35}S]dATPαS and 2 μl diluted T7 DNA polymerase. This was spun briefly to collect the contents together at the bottom of the tube, then incubated at room temperature for 5 min for the labelling reaction to proceed. As this incubation was taking place, the four sequencing mixes were placed at 37°C for at least 1 min to warm. After completing the labelling reaction, the termination reactions were carried out by adding 4.5 μl of the labelling reaction to each of the pre-warmed tubes, using a fresh pipette tip each time to avoid cross-contamination. The tubes were spun briefly before being incubated at 37°C for 5 minutes. Stop solution (5 μl) was then added to each tube, and spun briefly again to collect the contents at the bottom. These reactions could then be stored at -20°C, for several days if necessary, until run on a sequencing gel.

2.2.15.3 Preparation of sequencing gel

The glass plates, spacers and combs used for the gel (Gibco BRL Model S2 system, 0.4mm spacers and sharkstooth combs)

were washed carefully in warm water containing 2% RBS 25 detergent (Chemical Concentrates Ltd.) to remove any adhering material, then rinsed in deionised water and allowed to air dry. (The glass plates were rested on polystyrene blocks, the proposed inner sides uppermost). When dry, the plates were vigourously rubbed with tissue soaked in ethanol (on the sides to be in contact with the gel), and polished dry with clean tissue. For each plate in turn, 1 ml of Repel Silane (Pharmacia) was pipetted onto the surface and quickly rubbed over the entire plate (to prevent the gel sticking to either plate). The plates were again ethanol polished. The two plates were assembled by laying the spacers in position along the sides of the smaller plate and placing the larger plate on top, then clamping it in place with spring clips along the sides. The bottom edge was taped first, using packing tape, then each side. The spring clips were left in place for the pouring and setting of the gel, and the plates were positioned at an angle so that the upper end was slightly raised.

The gel was prepared by placing 60 mls of acrylamide stock solution in a clean beaker, and adding (in order), 75 μl of TEMED and 196 μl of a freshly made 10% ammonium persulphate solution. This was mixed gently using a 20 ml plastic syringe, which was then used to add the gel between the plates. The gel was added at one corner until the level almost reached the top, then across the gel to completely fill the space between the plates, whilst avoiding bubbles. The combs were then quickly inserted, flat edge first, to a depth of 2 to 3 mm below the short plate. (If this proved difficult to do, ie. too tight a fit, the top spring clips on either side were briefly removed, then replaced after the combs were in.) The gel was left to set (approximately 1 hour), then placed in the electrophoresis apparatus after first removing the tape covering the bottom edge. TBE buffer was placed in the upper reservoir to a level above that of the small plate, and left to stand for 5 minutes to allow any leaks to be detected (either through the top gasket or along the sides of the gel). When satisfied that no leaking of buffer was occurring, buffer was also added

to the lower reservoir, and the gel left in this condition until pre-electrophoresis. In practice, gels were poured the evening before they were to be run.

2.2.15.4 Electrophoresis

The gel was first pre-electrophoresed at a set current of 50mA for between 30 mins and 1 hour before adding the samples. During this time a waterbath was heated to 75-80°C, and 3 µl aliquots of each reaction mix prepared in microfuge tubes, and spun briefly to collect the contents in the bottom of the tube. When the waterbath was ready and the pre-electrophesis completed, the power was turned off, and the combs removed from the gel. They were rinsed in tap water, then re-inserted in the opposite orientation, so that the teeth were pressing against (but not piercing) the top of the gel. A length of masking tape was affixed to the outside of the glass plates just above the wells, and sets of four even wells chosen for each template/primer and loading, and labelled accordingly (always using the order A C G T). A gap of 2 - 3 empty wells was left between each set of four. (Typically, two different primer/template combinations were run on the same gel, with three loadings of each). The aliquots of reaction mixes were denatured by heating to 75 - 80°C, during which time the first set(s) of wells to be used were rinsed out with buffer using a disposable syringe and needle. After denaturation, the samples were loaded immediately into the wells, the power reconnected, and electrophoresed until the dark blue dye (one of two in the Stop Solution) had reached the end of the gel, (typically 1 - 2 hours). A further set of aliquots were then denatured, and added to the appropriate wells, as before. A third loading was subsequently run in the same way.

2.2.15.5 Drying and exposure of gel

When electrophoresis was complete, the gel was removed from the apparatus, and the two glass plates carefully prised apart, leaving the gel lying on one of the plates. This was then lowered very carefully into a bath of 10% methanol / 10% acetic acid, where it was left for 20 mins to remove excess urea. Great care was taken again in removing the glass plate from the bath, in order to keep the gel lying flat on it. Two sheets of Whatman #1 filter paper were then individually placed over the gel, taking care not to trap any air bubbles. This then allowed the gel to be lifted off the glass plate, and laid down with the paper underneath it. A sheet of clingfilm was then placed over the other side of the gel, and any bubbles gently rubbed out with tissue paper. The edges of the filter paper and clingfilm were then trimmed with a sissors to 1 - 2 cm larger than the size of the gel, and the whole "sandwich" laid on a Biorad Slab Dryer (Model 483), with the clingfilm uppermost. This was then covered with the rubber sheet of the dryer, and a vacuum applied (the pump having been turned on some minutes previously to warm up). Once a vacuum could be seen to be pulling properly, and any air bubbles were removed from underneath the rubber, the temperature was switched to 80°C, and the timer set to two hours. After this time the dried gel was removed, taped into an autoradiography cartridge (by the paper edges), the clingfilm removed, and a sheet of Kodak X-OMAT-AR film placed over it (in darkroom). (This was done quickly because of the sensitivity of the film). Note was taken of which plate the gel had stayed on and a corner was cut on the film, both to establish the correct orientation later. Gels were usually exposed for 48 hours (or longer as the radiolabelled nucleotide aged), then developed using Kodak LX24 developer until bands were visible, rinsed in water, fixed in Kodax FX40 fixer (> 10 min), and rinsed again. Bases were then read off manually, from bottom to top.

2.2.15.6 Sequence analysis

Sequences were entered to a PC using the SEQAID™ program (Intelligenetics) which also allowed comparisons of the sequences. Files containing the sequences (in IG format) were then sent via vax1@dcu.ie to the Irish National Centre for Bioinformatics (INCBI) @acer.gen.tcd.ie for further analysis. Here the sequences were converted to GCG format (using "fromig"), and homology searching was carried out by BLAST (Altschul *et al.* 1990) using the "blast" option in GCG:The Wisconsin Package. Searching was against GenEMBL, a non-redundant DNA database. Translation of sequences was also carried out in GCG, using "map". Sequences showing homology were extracted from the databases using the QUERY program ("find" option).

Chapter 3 :

Uptake of methylamine by *Rhizobium meliloti* 1021

3.1 ASSAY RESULTS

3.1.1 The methylamine transport assay

The method chosen to investigate ammonium transport in *R. meliloti* was one involving the analogue methylamine, widely used by other workers for the same purpose (See Chapter 1 Section 3). A ^{14}C-labelled version of the compound was used, in a method based closely on that of Jayakumar and Barnes (1983). Cells were resuspended for the MA uptake assay based on the $O.D._{550nm}$ of the culture at harvest, (as described in Materials and Methods, Section 2.2.1). This was in order to have all cells in the same concentration range for assay, without having to wait until protein determinations had been carried out. These were later determined by the Bradford method, and used to adjust the uptake assay results. Cells grown on low and high ammonium respectively were initially used as positive and negative controls. Unfiltered samples acted as internal controls on methylamine concentration in each reaction, and background and ^{14}C standards (supplied with the liquid scintillation counter [LSC]) were included in each counting.

3.1.2 Calculation of ^{14}C methylamine uptake

Values for uptake of methylamine by cell suspensions were initially received from the LSC in counts per minute (cpm) per 100 μl of reaction. The cpm value for 10 μl of unfiltered reaction (equivalent to 9.6×10^{-2} nmol of ^{14}C-MA) was used to convert cpm to nmol MA, then the Bradford assay results for protein concentration were taken into account in order to arrive at a result expressed in nmol MA per mg total cell protein. Therefore for each data point:

$$\text{Uptake in nmol MA / mg total protein} = [S \times 0.96] / [C \times P]$$

where S = cpm of sample
C = cpm of unfiltered 10 μl control
P = protein concentration, mg/ml

3.1.3 Methylammonium uptake activity

Assay conditions were initially optimised for wild type *R. meliloti* 1021 grown on 0.5mM NH_4Cl as the sole nitrogen source, on the assumption that these were the conditions under which an ammonium uptake system would be necessary for the growth of the organism. Uptake was detected under these conditions, and comparison was made with cells grown under excess ammonium (20 mM NH_4Cl) (Figure 3.1). These two sets of conditions were used thereafter as positive and negative controls.

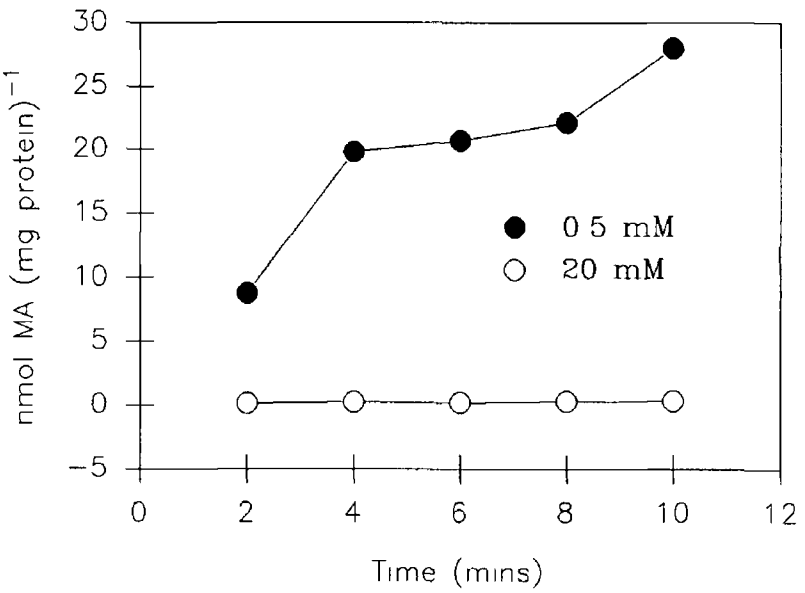


Figure 3.1. Uptake of methylamine by wild type *R. meliloti* 1021 grown on 0.5 mM or 20 mM NH_4Cl as sole nitrogen source.

3.1.4 Ability to utilise methylamine as a nitrogen source

It was important to know whether the strain being used could grow using the analogue (i.e. methylamine) as a nitrogen source, as it might therefore transport the compound for use itself, rather than simply because of its similarity to ammonium. Alternately, if methylamine were

toxic to the strain, it might provide a means of selecting an uptake mutant (by resistance).

Aliquots (40 μl) of a 5 ml overnight culture of *R. meliloti* 1021 in TY medium were used to inoculate 10 ml minimal broths containing either NH_4Cl or MA, at concentrations of 0.5 and 20 mM, as the sole nitrogen source. These were incubated with shaking at 30°C, and the growth followed by reading the absorbance at 550 nm. Table 3.1 shows the results obtained.

NITROGEN SOURCE FOR GROWTH	O.D. OF CULTURE AT 550 nm		
	17 hrs	40 hrs	64 hrs
0.5 mM NH_4Cl	0.730	0.851	0.716
0.5 mM MA	0.733	0.965	0.757
20 mM NH_4Cl	1.112	1.409	1.313
20 mM MA	0.770	1.102	1.200

Table 3.1 : Growth of *R. meliloti* 1021 on methylamine as sole nitrogen source.

These results show that *R. meliloti* 1021 can grow equally well on MA as a nitrogen source at low concentrations, although there may be a slight inhibitory effect at 20 mM. The possibility of using MA to select for a transport mutant is definitely ruled out, as it is not toxic at the concentration at which transport is likely to be occurring.

3.1.5 Inhibition of uptake by ammonium

The uptake activity (as first determined) appeared to be related to the presence or absence of ammonium, rather than methylammonium, in the growth medium. It was important to establish whether the system could actually carry ammonium, and whether it would do so in preference to methylammonium.

To this end, the inhibitory effect of ammonium in the actual assay was investigated. The effect of adding ammonium during the course of the assay is shown (Figure 3.2). This involved adding NH_4Cl to a concentration of 50 mM, but pre-incubation with 0.5 mM NH_4Cl was found to be sufficient to cause complete inhibition of MA uptake (not shown).

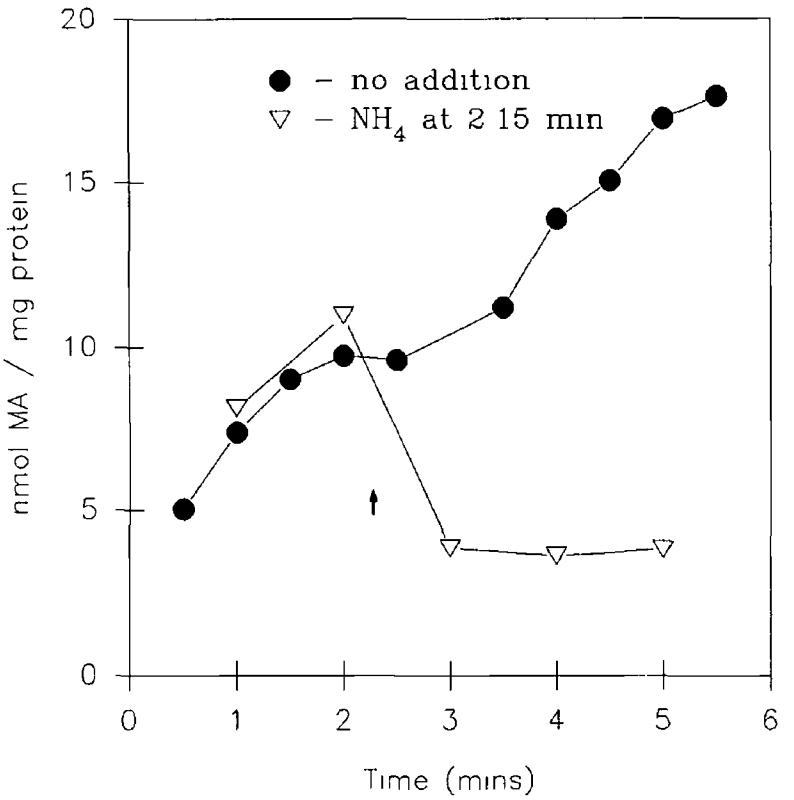


Figure 3.2. The effect of the addition of NH_4Cl on the uptake of methylamine by *R. meliloti* 1021.

There is a clear efflux of radiolabelled MA from the cells on the addition of ammonium, showing that not only is there competition for the same carrier, but there is also a pool of free (i.e. unmetabolised) MA within the cells.

3.1.6 Effect of using different nitrogen sources

The effect on uptake activity of growing the cells on other nitrogen sources was also investigated. The nitrogen sources investigated were KNO_3, glutamate, histidine, methylamine, glutamine and aspartate, and were used at concentrations of both 0.5 and 20 mM. The results are shown in Table 3.2, and can be compared to uptake when cells were grown on ammonium.

Nitrogen Source	Conc'n (mM)	nmol MA (mg protein)$^{-1}$(5 min)$^{-1}$	Percentage uptake
NH_4Cl	0.5	19	(100)
	20	0.2	1.2
KNO_3	0.5	15	80
	20	10	52
Glutamate	0.5	15	79
	20	5	27
Histidine	0.5	9	46
	20	0.1	0.4
Methylamine	0.5	13	70
	20	0.5	2.9
Glutamine	0.5	0.6	3.8
	20	0.5	2.6
Aspartate	0.5	16	85
	20	8.0	43

Table 3.2 : The effect of different nitrogen sources for growth on the subsequent MA uptake activity of cultures.

In general, for the nitrogen sources tested above, growth on a low concentration appears to stimulate uptake whilst growth on a high concentration leads to comparatively low uptake rates. The notable exception to this is glutamine, where even low levels of the amino acid in the growth medium appear to inhibit methylamine uptake activity.

3.1.7 Other inhibitors of the assay

Various nitrogen sources (and other compounds) were investigated to determine if they had an inhibitory effect on the assay itself. For this experiment, a culture was grown with 0.5 mM NH_4Cl as its sole nitrogen source, and the cells washed and resuspended as usual. Aliquots were then preincubated for 15 min at room temperature with the compound in question (at a concentration of 10 mM, except as indicated in Table 3.3), before being assayed for uptake of ^{14}C-MA. In addition to the nitrogen sources used previously, the glutamine analogues ethylenediamine (EDA) and methionine sulphoximine (MSX), and the metabolic inhibitor sodium azide were also investigated. Table 3.3 shows the relative inhibitory effects of these various compounds.

Compound	nmol MA (mg protein)$^{-1}$(5 min)$^{-1}$	Percentage uptake
Control	15	100
NH_4Cl	0.2	1.4
KNO_3	13	86
Glutamate	10	70
Histidine	9	65
Methylamine	0.1	0.6
Glutamine	0.2	1.3
Aspartate	13	90
EDA (0.2mM)	6	41
Sodium azide (5mM)	0.3	2.2
MSX (0.2mM)	0.3	2.0

Table 3.3 : The inhibitory effects of various compounds on the uptake of methylamine.

The compounds having the strongest effect in the assay are ones that also affected it strongly in the growth medium, namely NH_4Cl, MA and glutamine. Sodium azide is also a strong inhibitor, as is MSX, despite its low concentration.

3.1.8 Induction of uptake activity

As shown previously, low levels of uptake were observed in cells initially grown in 20 mM NH_4Cl as the nitrogen source. However when the same cells were washed, transferred to low nitrogen medium and incubated overnight, it was observed that uptake activity was induced. Transfer to nitrogen-free medium also resulted in induction of uptake.
Figure 3.3 shows the onset of uptake activity in a culture originally grown on 20 mM NH_4Cl, then transferred to minimal medium lacking a nitrogen source, and samples taken for assay from this point onwards.

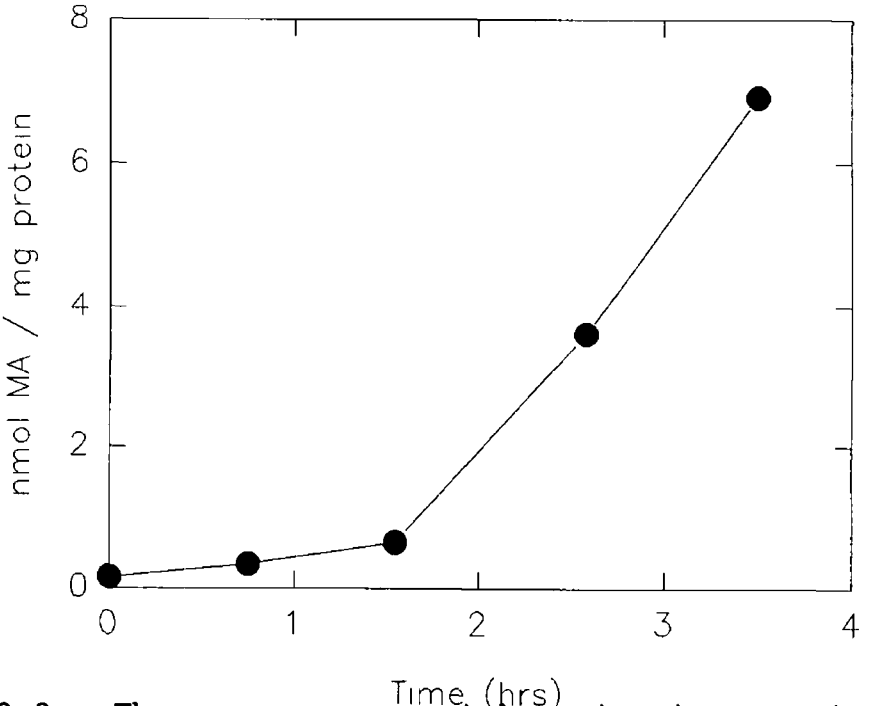


Figure 3.3 : The increase in uptake activity over time of a culture initially grown on 20 mM NH_4Cl, then washed and transferred to nitrogen-free medium.

3.1.8.1 Inhibition of induction

An experiment was designed to discover whether this induction involved regulation at a genetic level, i.e. the expression of a gene. Chloramphenicol was added to assess the effect of inhibiting protein synthesis on the induction of methylammonium uptake. Four cultures were set up, two initially grown with 0.5 mM NH_4Cl and two with 20 mM NH_4Cl as sole nitrogen source. In each case the cells were then washed and resuspended in low nitrogen medium, with chloramphenicol being added to one of each original type. A four hour incubation was found to be sufficient to induce transport activity in a culture originally grown with 20mM NH_4Cl, without the antibiotic causing appreciable decrease in the activity of the control cells (grown at 0.5mM). Figure 3.4 shows uptake activity for the four cultures, firstly after their initial growth, then later after the resuspension/induction process, showing clearly the inhibitory effect of chloramphenicol. When the cells grown on 20mM NH_4Cl and exposed to chloramphenicol (i.e. the uninduced culture) were subsequently washed and resuspended again in fresh low nitrogen medium without the antibiotic, uptake activity was induced to the control level (15 nmol MA / mg protein / 5 min).

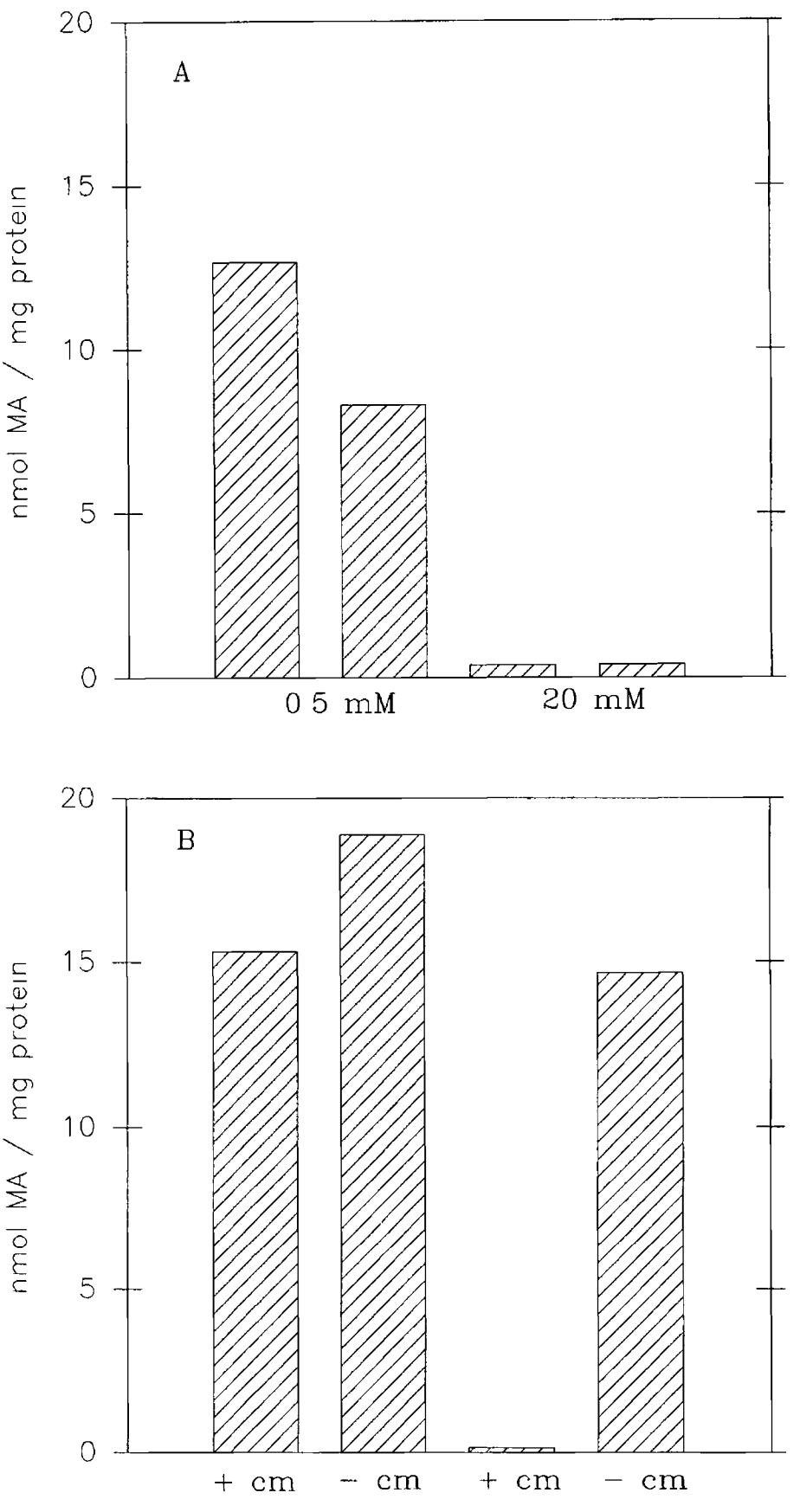


Figure 3.4 : The inhibition by chloramphenicol of the induction of uptake activity. A: Initial uptake activities (per 5 mins), before induction. B: Activities following induction / addition of chloramphenicol.

3.1.9 Uptake activity in *ntr* and *gln* mutants

Strains carrying mutations in the *ntrA* and *ntrC* genes (involved in nitrogen regulation) were assayed for uptake activity after being grown on the same minimal medium as the wild type.
Mutants in the *glnA, glnII* and *glnT* genes (encoding the three glutamine synthetases identified in *R. meliloti*), and a double mutant, *glnAglnII*, were likewise grown and assayed, with culture samples being taken at the time of assay and checked on the appropriate antibiotics to insure maintenance of the mutant phenotype. The *glnAglnII* double mutant showed poorer growth than the wild type on minimal medium, only reaching an $O.D._{550nm}$ of approximately 0.6 compared to 0.8 - 0.9 for the other mutants and the wild type. Nevertheless the culture retained its antibiotic resistances (Km and Gm) in 100% of cells, as did the other *gln* mutant strains. This was shown by plating the cultures used for the uptake assay onto LB agar plates to get separate colonies, then replica plating these onto fresh LB and also LB plus the appropriate antibiotics. Strain 1021 (wild type) was included as a control. Approximately 100 colonies were replica plated in each case. Interestingly, the double mutant had been previously characterized as being a glutamine auxotroph (de Bruijn *et al.* 1989), but this was not found to be the case in this work. The *gln* mutant strains were also grown on rich medium (TY) and induced for transport activity, and similar uptake results were found.

Figure 3.5 shows the uptake results for the *gln* mutants when grown both on minimal medium or on rich medium (and activity induced). Table 3.4 lists the results for all the mutants tested, both *ntr* and *gln* mutants, all grown on minimal medium.

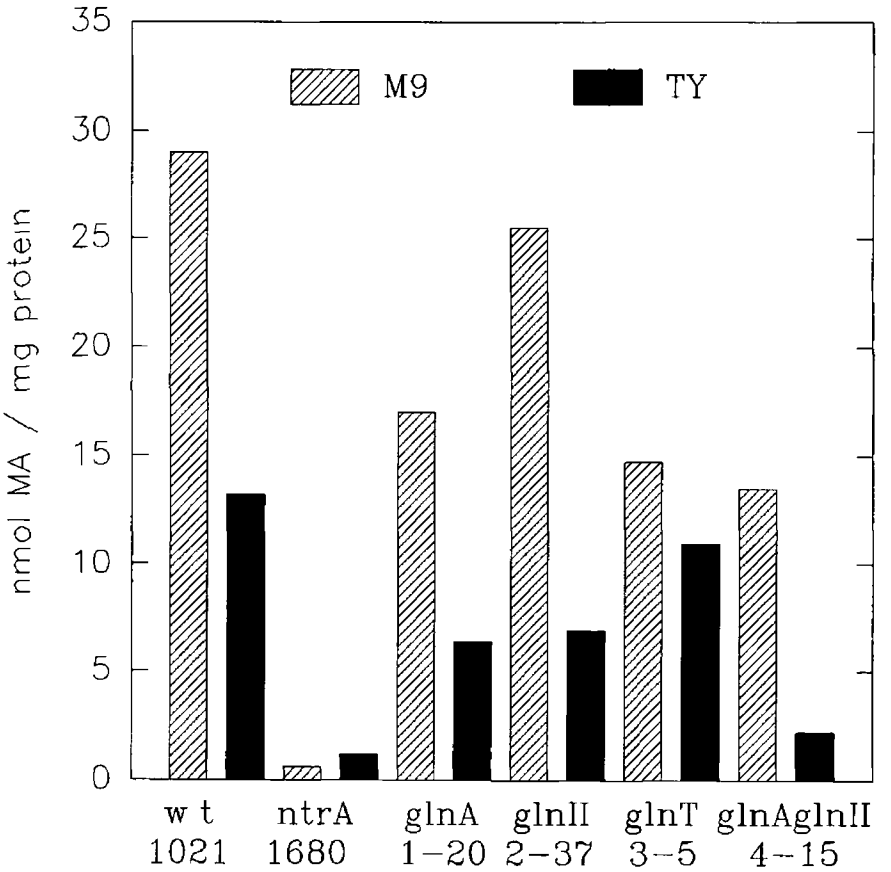


Figure 3.5 : Uptake of methylamine by mutant strains grown on 1) minimal medium and assayed directly, 2) TY medium, washed and transferred to nitrogen-free medium to induce uptake and subsequently assayed.
(These results are average values obtained in three seperate experiments.)

R. meliloti Strain (mutation)	nmol MA (mg protein)$^{-1}$ (5 min)$^{-1}$	% Uptake
1021 (wild type)	(0.5mM) 23 (20 mM) 0.17	(100) 0.7
1680 (*ntrA*)	1.0	4.5
5001 (*ntrC*)	0.2	0.7
5003 (*ntrC*)	0.8	3.5
5422 (*ntrA*)	0.8	3.7
G649 (*ntrC*)[3]	(0.5 mM) 24 (20 mM) 1.4	104 6.1
1-20 (*glnA*)	9.4	41
2-37 (*glnII*)	12.6	55
3-5 (*glnT*)	18.4	80
4-15 (*glnAglnII*)	10.0	43

Table 3.4 : MA uptake results for various strains grown on minimal medium with 0.5 mM NH_4Cl as sole nitrogen source, (except as indicated for strains 1021 and G649).

There is a striking lack of uptake activity in strains lacking functional NtrC (*ntrC*) or σ^{54} (*ntrA*), but no such dramatic effect for mutations in the *gln* genes.

[3] This strain is reported to constitutively activate several σ^{54}-dependent promoters (Labes *et al.* 1993).

3.1.9.2 Growth of mutants lacking uptake activity

The *ntr* mutants which did not exhibit uptake activity grew as well as the wild type on 0.5 mM NH_4Cl as the nitrogen source, judging by similar O.D.s of the cultures at harvesting. Their growth on methylamine as sole nitrogen source was also compared with the wild type, to see if the loss of uptake activity impaired the assimilation of methylamine but not ammonium. Table 3.5 compares the growth of mutant strains 1680 (*ntrA*) and 5001 (*ntrC*) with wild type 1021 on NH_4Cl and on MA as the sole nitrogen source, and as can be seen, the lack of uptake activity does not correlate with any apparent deficiency in assimilation.

	O.D.$_{550nm}$ after 40 hours			
Strain	0.5 mM NH_4Cl	0.5 mM MA	20 mM NH_4Cl	20 mM MA
1680	0.867	0.950	1.477	1.187
5001	0.978	1.205	1.533	1.178
1021	0.851	0.965	1.409	1.102

Table 3.5 : The growth of *ntr* mutants on NH_4Cl and MA as sole nitrogen source, compared with the wild type strain.

3.2 DISCUSSION

There is a clear difference in the ability of wild type cells grown on different nitrogen sources to accumulate radiolabelled MA as assayed by this method. This is very noticeable particularly for different concentrations of ammonium, where growth on high levels (20 mM) appears to be inhibitory, whilst use of a low concentration (0.5 mM) gives the highest activity found. In general, growth on lower concentrations of nitrogen appears to promote uptake, with the exception of growth on glutamine, where even use of 0.5 mM as the sole nitrogen source results in very low levels of uptake activity in the culture (Table 3.2). Effects of nitrogen sources in the assay itself echo these results, with glutamine analogues (EDA and MSX) also exerting a strong inhibition (Table 3.3). These results are similar to the "nitrogen control" effect found previously for methylamine uptake systems in other organisms, although the inhibitory effect of glutamine has varied from species to species (See Chapter 1 Section 1.3.3). Inhibition of uptake by sodium azide has also been previously found for several organisms, and is taken as an indicator of a requirement for respiratory energy.

The fact that *R. meliloti* 1021 can, unlike some other bacteria, utilise methylamine as a nitrogen source (Table 3.1) makes it possible that a specific uptake system exists for the analogue itself, rather than an ammonium system that accepts both compounds. However, ammonium certainly shares the same carrier - as shown by its ability to cause efflux of labelled MA (Figure 3.2) - and the transport activity is not specific to growth on methylamine. This suggests an ammonium carrier accepting methylamine, especially as there appears to be a slight inhibitory effect on growth by high concentrations of MA (Table 3.1), making it unlikely to be a preferred source of nitrogen. Indeed the induction of uptake appears not to be triggered specifically by a low external concentration of ammonium (0.5 mM, which is sufficient in fact to inhibit uptake) but

by a lack of nitrogen in the surrounding medium (shown by the induction of uptake using nitrogen-free medium), and is probably a response to depletion of the nitrogen source. The fact that this response is inhibited by chloramphenicol (Figure 3.4) indicates the synthesis of a polypeptide, i.e. control at the genetic level.

The inability of strains lacking functional σ^{54} and NtrC (mutants in *ntrA* and *ntrC*) to accumulate methylamine under conditions where the wild type can do so (Table 3.4), indicates a process regulated by the Ntr system. This finding has been made previously for another member of the *Rhizobiaciae*, namely *Bradyrhizobium (Parasponia)* sp. ANU289, where an *ntrC* mutant was found to be lacking in ammonium transport activity (Udvardi *et al.* 1992). The latter mutant was isolated on the basis of comparatively poor growth at a concentration of ammonium that prompted transport activity in the wild type, based on the assumption that the transport system was a requirement for good growth. However the *ntr* mutants examined in this work are striking for their ability to grow equally as well as the wild type in conditions where the wild type expresses uptake activity. Not only is this distinct from the situation in ANU289, but it shows that contrary to expectation, the system assayed is not required for growth under these conditions. This raises questions both as to its purpose, and to how the mutant cells are therefore acquiring ammonium in these circumstances. One possibility is that the organism possesses another ammonium transport system, one that cannot also accept methylamine and therefore is not measured by this assay, and is not regulated by the Ntr system either. Whatever the explanation, this appears to rule out the possibility of selecting a transport mutant by means of the strategy employed for the *ntrC* mutant of ANU289.

There has been difficulty in previous work in separating the transport of ammonium from its subsequent assimilation (See Chapter 1 Section 3). In particular, this has focussed

on the contribution made to overall uptake by the enzyme glutamine synthetase. It was for this reason that the *gln* mutants of strain 1021 were assayed for uptake of methylamine, although the presence of three GS enzymes in this strain makes the situation more complex. However, if there is a strong GS component in the overall uptake effect, this appears not to be linked specifically to any one of the three genetic loci *glnA, glnII* or *glnT* (Figure 3.5, Table 3.4). Two of these are known to be under Ntr control (*glnA* and *glnT*), but judging by the assay results obtained here, neither could be directly responsible for the lack of activity seen in the *ntrA* and *ntrC* mutants, as any reduction of uptake activity in the former is negligible compared to that in the latter. Therefore the effect on activity of a deficiency of NtrC cannot be solely due to an effect on *glnII* or *glnT* (if indeed it is influenced at all by these two genes), but must involve at least one other gene under Ntr control.

The *glnAglnII* double mutant cannot be assumed to be lacking all active GS (despite results reported previously, de Bruijn *et al.* 1989), as it seems to exhibit ammonium assimilatory ability, albeit at a lesser level than the other mutants or the wild type. Therefore all the mutants assayed here do have at least some assimilatory ability, though derived from different genetic loci, so an assimilatory component to overall uptake cannot be ruled out. Neither can the inhibitory effect of MSX be ascribed totally to inhibition of a transporter. It may also be inhibiting the assimilatory activity of GS. It is worth noting also however that the efflux of labelled MA from transporting cells by the addition of ammonium (Figure 3.2) indicates a pool of free unmetabolised MA within the cells, despite the ability of strain 1021 to utilise the analogue. This suggests distinct steps of transport and assimilation, rather than rapid assimilation causing diffusion into the cell.

Previous characterization of methylammonium transport in *R. meliloti* described a system with a maximum uptake rate of 10 nmol / min / mg protein (Pargent and Kleiner 1985). The results shown here, although not characterised in terms of Michaelis-Menten kinetics, appear to be in the same range of activity. There is agreement also in the effect of ammonium, and in the "nitrogen control" observed previously, also in the same work.

Chapter 4 :

The isolation of putative σ^{54}-controlled genes

4.1 The isolation of DNA containing the σ^{54} promoter

4.1.1 Introduction

The finding that active ammonium uptake is not essential for growth, (as detailed in Chapter 3), ruled out the isolation of a mutant in ammonium uptake by selecting for an impairment in or lack of growth on low ammonium, an approach attempted previously with *Bradyrhizobium* sp. ANU289 and resulting in the isolation of an *ntrC* mutant (Udvardi *et al.* 1992). Clearly this would not work for the strain used here, since the *ntr* mutants shown to lack transport activity (as measured by methylammonium uptake), were unaffected in their ability to grow on 0.5 mM NH_4Cl. Neither was selecting by resistance to the analogue (MA) an option, as it can be utilised as a sole nitrogen source and did not appear to inhibit growth to any marked extent, even at 20 mM, a concentration which was too high for expression of the uptake activity anyway. Therefore neither of the above approaches could be used to isolate a mutant, and hence identify (by complementation) a specific transport gene. However the requirement for σ^{54} in the expression of the system suggested some interesting possibilities for the isolation of whatever gene was being directly transcribed by RNA polymerase with the sigma factor. The promoter sequence at which σ^{54} has been shown to bind is highly conserved, even between species (See Section 1.4.4, Table 1.1). This suggests a possible site for probing or, (as in this work), a priming site for a PCR reaction, amplifying the gene. However, the problem of the second necessary priming site downstream from the σ^{54} promoter had to be overcome. This was achieved as follows. A gene bank of 1.5 - 2.5 kb fragments of genomic DNA was constructed in the vector pUC18. This plasmid contains sites on either side of its multiple cloning site to which commercially produced primers are available. Thus a plasmid containing an insert with a σ^{54} promoter sequence could act as a template for PCR amplification of the DNA sequence starting at the promoter, going downstream into the gene until the end of the insert, on into the multiple cloning site of the vector, and

finishing with the vector primer sequence. By selecting a vector primer to either side of the multiple cloning site, both possible orientations of the insert could be used. Subsequent cloning and sequencing of the PCR products should then show the starting sequences of various genes with σ^{54} promoters, i.e. under Ntr control. Figure 4.1 shows an outline of this strategy.

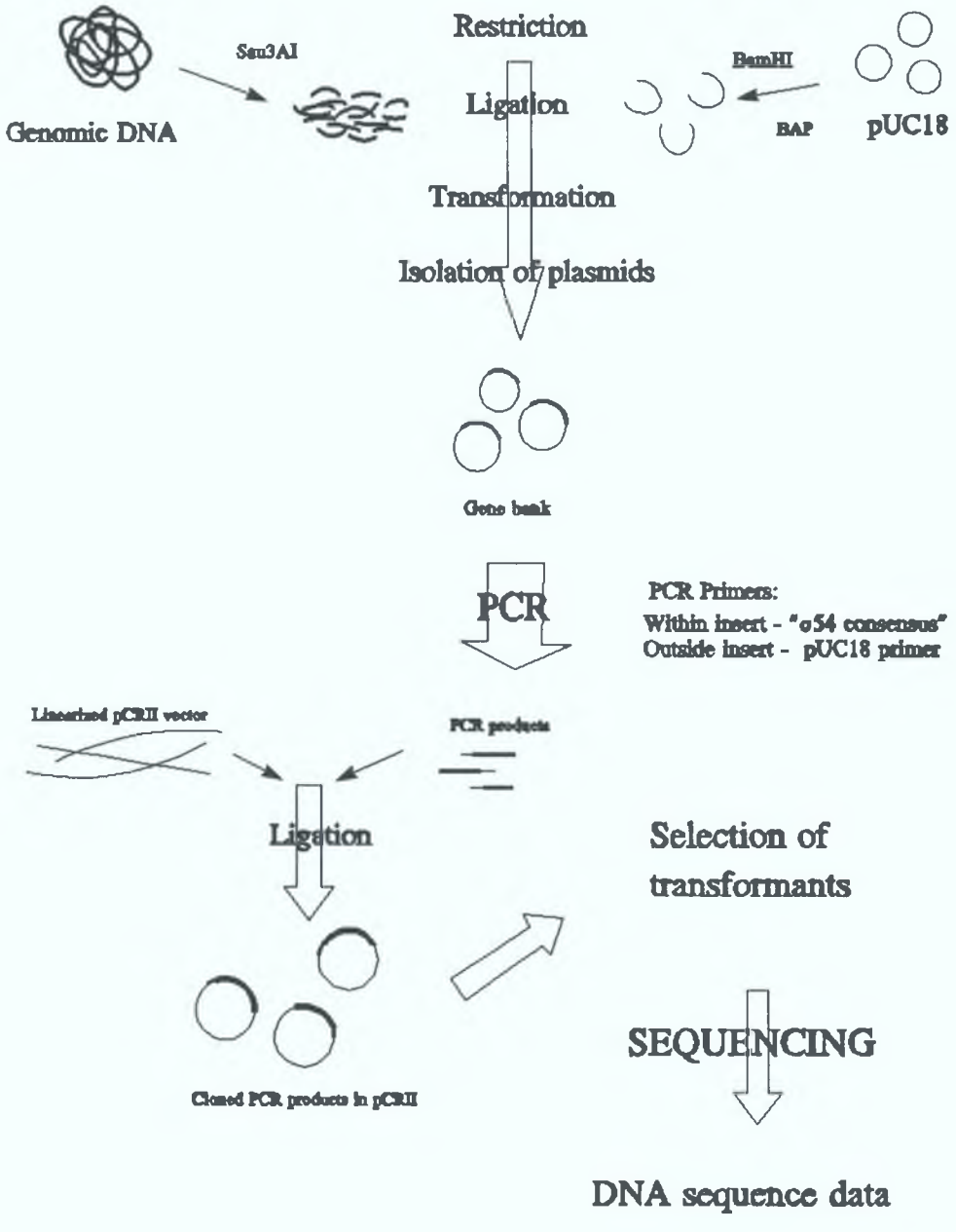


Figure 4.1 : The experimental strategy used to isolate fragments of DNA containing σ^{54} promoter sequences. The choice of primer sequences for PCR is explained in Section 4.1.3.2 below.

4.1.2 Creation of an *R. meliloti* gene bank

4.1.2.1 Partial digestion by *Sau*3AI

Total DNA was isolated from *R. meliloti* strain 1021 as described in Materials and Methods. Partial digestion by the restriction endonuclease *Sau*3AI was used to obtain DNA fragments in the size range 1.5 - 2.5 kb. It proved difficult to select concentrations of DNA and enzyme giving this size range, as apparent slight changes in concentrations (possibly due to pipette error or incomplete mixing) resulted in either too much or too little digestion. This was solved by mixing a bulk reaction, (270 µl DNA, 294 µl sterile water, 63 µl React 4™ 10X buffer, 3 µl *Sau*3AI), and taking a 35 µl aliquot of this which was incubated at 37°C, and samples taken every 10 min. These were later compared with standard markers on a 0.8% agarose/TAE gel. The optimum incubation time was then chosen, and the remaining aliquots incubated for this period. The resulting digestions were subseqently run on a 0.8% agarose/TAE gel, the appropriate portion of the gel corresponding to the size range 1.5 - 2.5 kb excised, and the DNA isolated. The concentration of the isolated DNA was estimated (by comparison with known standards) to be approximately 0.05 µg/µl.

4.1.2.2 Ligation

The vector chosen was a commercial preparation of pUC18, restricted with *Bam*HI, and also treated with phosphatase (BAP) to prevent recircularisation (Pharmacia, 27-4855-01). This was resuspended (from lyophilised) to 0.05 µg/µl with TE buffer. Vector and insert were mixed in a 1:1 ratio to a total of 1 µg, and ethanol-precipitated to reduce the volume. The precipitated DNA was resuspended into a 5 µl ligation reaction (i.e. 3 µl sterile water, 1 µl 5X ligase buffer and 1 µl T4 DNA ligase) and incubated overnight at 20°C. This ligation was subsequently repeated using double

the amounts of vector and insert DNA.
The efficiency of ligation was checked by electroporation of *E. coli* DH5α with the ligation mix, and plating onto LB containing ampicillin and X-gal, scoring for white colonies.

4.1.2.3 Results of ligation

Size (μg DNA)	White colonies per 100 μl transformation	Electroporation efficiency
1	> 12	9.5×10^6
2	424	2.5×10^7

Table 4.1 : Plate count results of ligations.

The number of colonies with plasmid containing an insert for the second ligation indicates 4,240 transformants per ml of transformed cells. Given that the size of the genome is approximately 4,000 kb, and the insert size is approximately 2 kb, this ligation alone should be sufficient to cover the entire genome. This ligated DNA was therefore used in subsequent manipulations.

The ligations could not be used directly as template DNA in the PCR reaction because the use of a dephosphorylated vector gives nicks in the DNA at either side of the insertion. As the PCR was designed to have one primer priming from within the insert and one from pUC18, the gaps would be within the fragment for amplification, interrupting the *Taq* DNA polymerase activity and thereby stopping the amplification before it had properly begun. Therefore it was necessary to repair the DNA by introducing it into electrocompetent cells, and allowing the cells to carry out the repair of the gaps.

4.1.2.4 Transformation

Electroporation was used to transform *E. coli* DH5α electrocompetent cells with the ligation mix, using 2 μl of ligation per 100 μl of competent cells. The resulting 1 ml cultures from each separate electroporation were added (after the initial 37°C recovery period) to 5 ml of fresh LB broth containing 25 μg/ml ampicillin, and incubated (shaking) at 30°C for 48 hours. Plasmid DNA was then isolated from these cultures using the STET method.

4.1.3 Polymerase Chain Reaction

4.1.3.1 Background

Polymerase chain reaction (PCR) is a technique for the amplification of specific stretches of DNA, the ends being specified by homology to the two primers used in the reaction. A cycle of denaturation (of double stranded DNA), annealing (of primer to template) and elongation (of the primers from their 3' ends) is repeated 20 - 30 times, each step being defined in terms of the length of time at the appropriate temperature. The use of a thermostable DNA polymerase such as that from *Thermus aquaticus* eliminates the problem of enzyme degradation during the denaturation step (typically at 90 - 95°C). Elongation (or extension) is usually at 70 - 75°C, depending on the optimum temperature of the enzyme, while the annealing temperature depends on the oligonucleotide composition and length of the primers. The area of DNA between the binding sites of the two primers becomes amplified in an exponential manner, yielding a double stranded product that includes the appropriate primer sequences on either end.

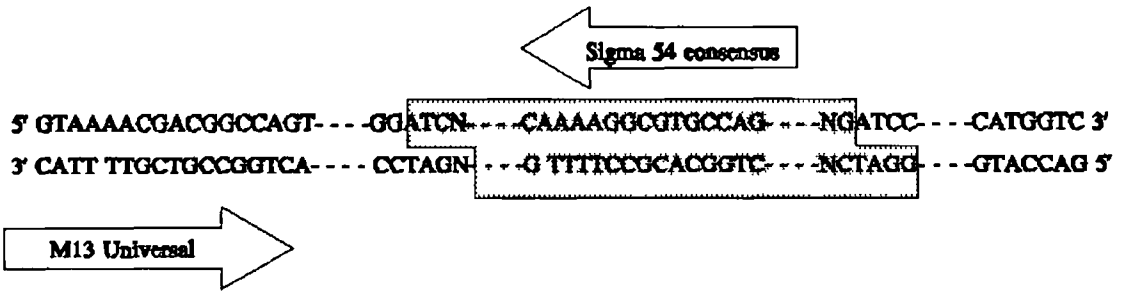
4.1.3.2 Choice of primers

Two primers, one specific to either side of the multiple cloning site of pUC18, were chosen from those commercially available, namely the M13 Universal primer and the T7/T3α primer. This was to allow priming from either side of the insert. Another primer was designed to prime within the insert, i.e. to any σ^{54}-specific promoter site. This primer was designed based on the sequences shown in Table 4.2, and synthesized by R&D Systems Europe Ltd. Figure 4.2 shows how the PCR was designed to proceed depending on the orientation of the σ^{54} promoter site in the insert.

glnII	C G A G T T G G C A C G T T T G A T G C T T A A G G C A A A T G G	Shatters *et al.* 1989
dctA	C A A A C T G G C A C G C A T G T T G C T G A C A A G C T C C A C	Engelke *et al.* 1989
nifHDK (P1)	A C G G C T G G C A C G A C T T T T G C A C G A T C A G C C C T G	Sundaresan *et al.* 1983a
fixABCX (P2)	G A C C T G G C A C G A C T T T T G C A C G A T C A T C C C C C	Earl *et al.* 1987
fixF	A A A A T T G G C A C G A G T T T T G A G A T T C C C G G T C A T	Aguilar *et al.* 1987
mos	C G G C C T G G C A C G C C T T T T G C A C G A T C A G C C C T G	Murphy *et al.* 1988
nfe	A A A G A T G G G A A T G C C T T T G C A T C G A G G C G T T G G	Sanjuan and Olivares 1989
P3	C G G C T T G G C A C G A C T T T T G C A A G A T C A C C C A G T	Better *et al.* 1983
nifB	G G A T T T G G C A T A G C T G T T G C T G G T T G A A T T G C A	Buikema *et al.* 1987
	C T G G C A C G C C T T T T G	σ^{54} consensus primer

Table 4.2 : Promoter sequences for *R. meliloti* genes under σ^{54} control, and the σ^{54} consensus primer sequence designed for this work.

1st orientation:



2nd orientation:

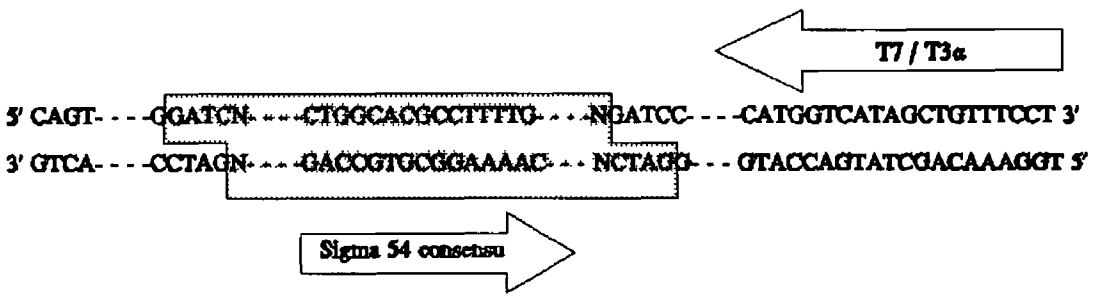


Figure 4.2 : The PCR reaction. The above shows an insert (shaded box) with *Sau*3AI ends and containing a σ^{54} promoter sequence, inserted into the *Bam*HI site of the vector pUC18. Both possible orientations of the insert are shown and the primers necessary for amplification in each case. The PCR products will therefore include the promoter sequence, the remainder of the insert downstream from the promoter, followed by vector DNA and finally the vector primer sequence.

The annealing temperature for each primer was calculated using the following equation:

$$T_a = T_m - 5\ °C = [\ 2(A + T) + 4(G + C) - 5\ ]\ °C$$

where T_a = annealing temperature,
T_m = melting temperature,
A,T,C,G = number of particular base in primer.

Primer	Sequence (5' - 3')	T_a
M13 Universal	G T A A A A C G A C G G C C A G T	47
T7/T3α	A A C A G C T A T G A C C A T G	41
σ^{54} consensus	C T G G C A C G C C T T T T G	43

Table 4.3 : Primers selected for the PCR amplification of σ^{54}-dependent genes, and their calculated annealing temperatures.

The concentration of each primer to be used was chosen as 0.5 μM. The M13 Universal and T7/T3α primers were supplied at 5 pmol/μl, and the "σ^{54} Consensus" oligonucleotide at 0.2 μmol in 500 μl. Concentrations of 1.5 mM $MgCl_2$ and 0.25 mM of each dNTP were chosen, in a total reaction volume of 50 μl.

The reaction components were therefore as follows:

10X buffer		5 μl
$MgCl_2$ (25 mM)		3 μl
Each dNTP (10 mM stock)		1.25 μl
Template DNA		1 μl
M13 Uni./T7/T3α primer		5 μl
σ^{54} primer (1/40 diln.)		2.5 μl
Taq DNA Polymerase		0.5 μl
Sterile dH_2O	to	50 μl

4.1.3.3 Thermocycler program

The following program was used:

Initial denaturation	-	94°C for 5 min

Followed by 30 cycles of:

Denaturation	-	94°C for 1 min
Annealing	-	38°C for 2 min
Extension	-	72°C for 2 min

4.1.3.4 PCR using plasmid miniprep as template

Reactions were set up with both combinations of primers (i.e. M13 Universal and σ^{54} Consensus, T7/T3α and σ^{54} Consensus), and also controls, one containing no enzyme, and one no template DNA.
A 5 μl sample of each completed reaction was subjected to electrophoresis on a 4% Nusieve agarose gel alongside suitable markers (123 bp ladder, BRL).

Plate 4.1 shows the PCR products formed with the template DNA and the T7/T3α and σ^{54} Consensus primers. No bands were observed for the alternative combination of primers (i.e. M13 Universal and σ^{54} Consensus) or for the controls.

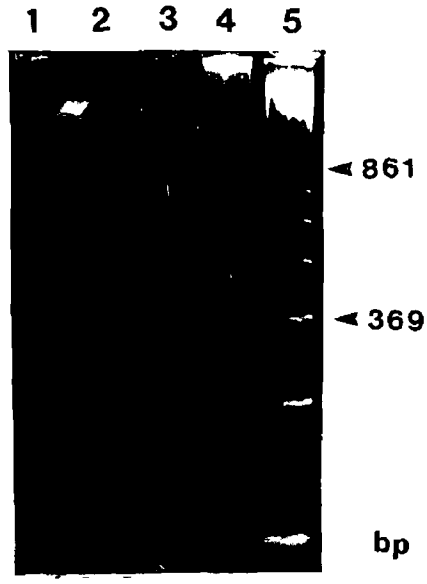


Plate 4.1 : Nusieve agarose gel (4%) of completed PCR reactions.

Lane 1: control - no enzyme
Lane 2: control - no DNA
Lane 3: PCR reaction using template DNA (1/50 dilution) and T7/T3α and σ^{54} Consensus primers.
Lane 4: Template DNA (undiluted)
Lane 5: Markers

Attempts to repeat these results were initially unsuccessful. In an attempt to decrease the specificity of the reaction, the annealing temperature was reduced, first to 37°C, then to 36°C. The concentrations of the reaction components were the same as originally, and the template was used at a 1 in 5 dilution. The 37°C reaction yielded nothing, however at 36°C, bands were observed, but not as many as previously.

Changes in the concentration of the dNTPs and $MgCl_2$, and a further reduction in the annealing temperature enabled other bands to be amplified (Plate 4.2).

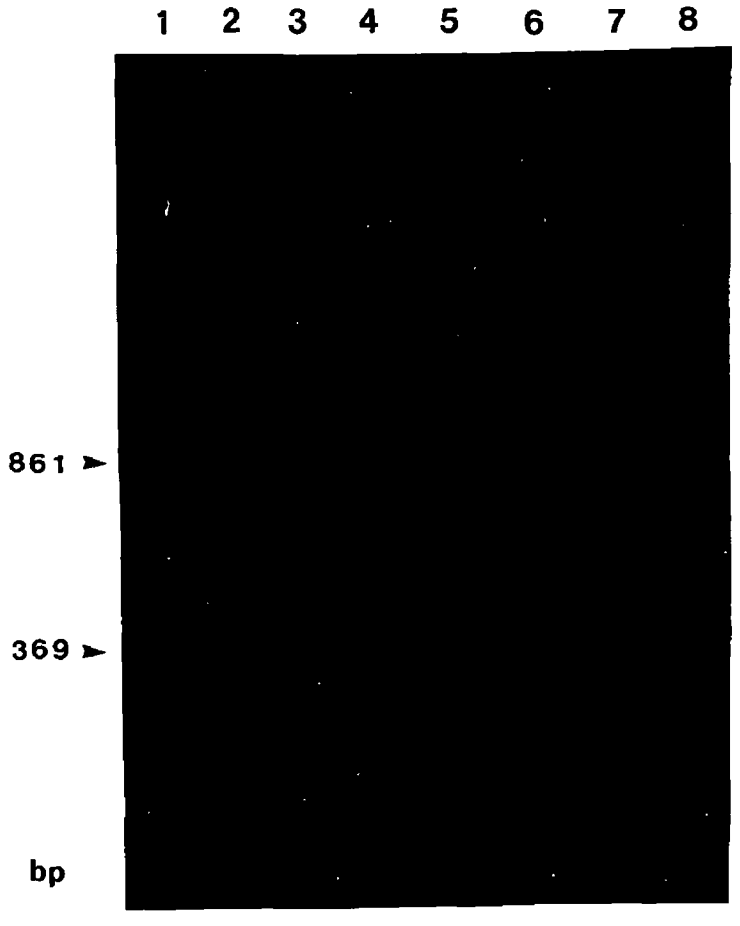


Plate 4.2 : Nusieve agarose gel (2%) of PCR reactions carried out at 35°C and subsequently used in cloning.

Lane 1: Markers
Lane 2: Control with no template DNA, 1.5 mM $MgCl_2$
Lane 3: template DNA (as previously) 1.5 mM $MgCl_2$
Lane 4: as Lane 3 except 3.0 mM $MgCl_2$
Lane 5: a previous positive reaction as template (i.e. positive reactions that gave 1 - 2 bands in the 250 - 350 bp range, pooled, extracted with chloroform to remove the paraffin and used undiluted), 1.5 mM $MgCl_2$
Lane 6: As Lane 5 except 3.0 mM $MgCl_2$.
Lane 7: Undiluted plasmid miniprep template, 1.5 mM $MgCl_2$
Lane 8: As Lane 7 except 3.0 mM $MgCl_2$.
(A band at approximately 300 bp appears in the control reaction to which no template DNA had been added.)

4.1.4 Cloning of PCR products

Those reactions showing positive for PCR products (seen in Lanes 4,5 and 7 in Plate 4.2) were used for cloning with the TA Cloning Kit (Invitrogen). This kit exploits a property of *Taq* DNA polymerase - that of adding a single deoxyadenosine to the 3' ends of PCR products - by utilising a linearized vector with single 3' deoxythymidine overhangs. The vector is engineered to have *Eco*RI sites on either side of the PCR-cloning site, and various primer sites to allow sequencing of the insert from either direction (See Figure 4.3).

The PCR products being cloned must be fresh to avoid degradation of the 3' overhangs, so the chosen PCR reactions were ligated to the pCRII vector within 24 hours of the PCR. These ligations were subsequently used to transform the competent cells supplied in the kit (*E. coli* INVαF'). Subsequent plating (on LB containing kanamycin and X-gal) gave the following results:

PCR reaction #	Colonies per 100 μl	
	blue	white
4*	0	7
5	12	30
7	10	29

Table 4.4 : Transformation results from cloning of PCR products - number of colonies per 100 μl transformed cells.

* This ligation took place approximately 14 hrs after the other two

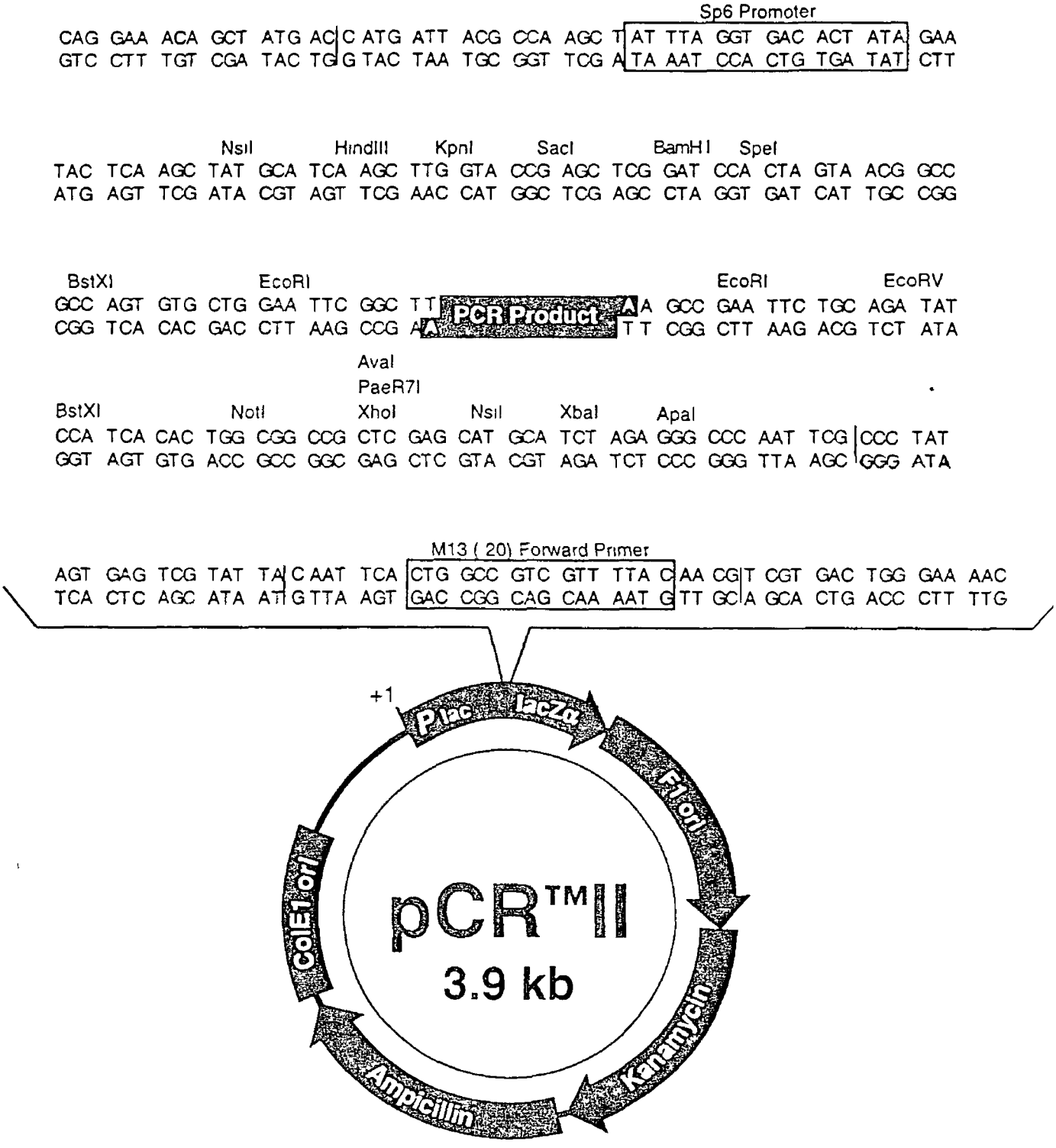


Figure 4.3 : The pCRII vector.

4.1.5 Restriction analysis of transformants

A total of 58 transformants were selected, grown in 5 ml LB / kanamycin, and their plasmid DNA isolated by the STET miniprep method. These plasmids were then digested with *Eco*RI restriction endonuclease (to excise the insert), and the results compared on a 2% Nusieve agarose gel. The object of this procedure was to identify distinct PCR products on the basis of size difference before commencing to sequence these.

The largest inserts found came from PCR reaction #7: unsuprisingly given the product sizes from the reaction. The predominant size was approximately 700 bp, but inserts in the range 200 - 300 bp were also common. One insert of approximately 500 bp was also isolated, the only one of this size amongst the three PCR reactions. Reaction #5 only gave inserts in the range 200 - 300 bp, but #4 also gave larger cloned products, up to approximately 400 bp. Plates 4.3 and 4.4 show the different sizes of inserts in several transformants.

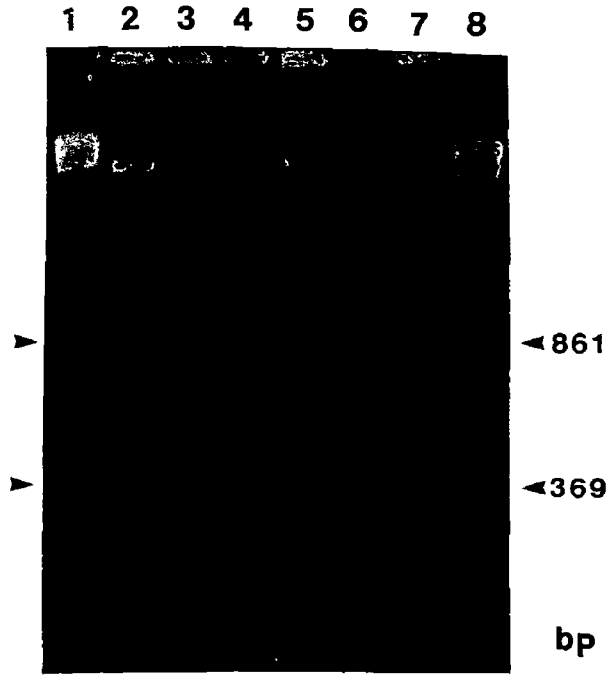


Plate 4.3 : Nusieve agarose gel (2%) of *Eco*RI digests of plasmids containing cloned PCR products.

Lanes 1 : Markers
Lanes 2 - 7: Transformants 42, 31, 1, 6, 45 and 50 respectively
Lane 8 : Markers

(Digestions were found to be partial in all cases but generally yielded a visible amount of insert DNA.)

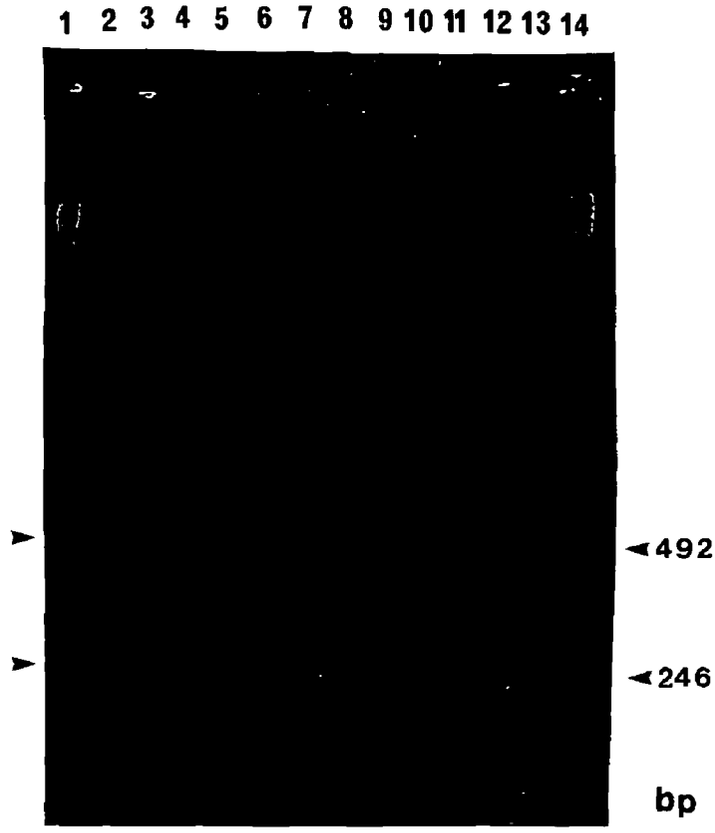


Plate 4.4 : Nusieve agarose gel (2%) of *Eco*RI digests of plasmids containing cloned PCR products.

Lanes 1 : Markers

Lanes 2 - 13: Transformants 32, 8, 17, 26, 40, 46, 52, 53, 54, 55, 30 and 42 respectively

Lane 14 : Markers

(Digestions were found to be partial in all cases but generally yielded a visible amount of insert DNA.)

The following are the transformants finally chosen to proceed with:

Transformant Number	Approx. size of insert (bp)
1	590
6	775
30	370
31	525
42	465
45	490
54	310
55	340

Table 4.5 : Transformants chosen for sequencing.

4.1.6 Sequencing of inserts

Plasmids to be sequenced were purified by the Qiagen column method, and the concentration of the DNA estimated by comparison on an agarose gel with a known concentration of pUC18. A 2 μg aliquot of DNA was used per set of sequencing reactions. The pCRII vector contains annealing sites for the M13 Universal primer (also called M13 -20 Forward) and the SP6 Promoter primer allowing an insert to be sequenced from either direction (See Figure 4.3). Initially, the primer used was the M13 Universal primer supplied with the sequencing kit. However when sequencing in this direction revealed a PCR product beginning with the T7/T3α primer rather than the σ^{54} consensus primer, the insert in question was then sequenced from the other end using the SP6 Promoter primer. This was is order to sequence the region directly downstream from the σ^{54} promoter. It was necessary

to use the SP6 Promoter primer for numbers 55 and 1. All eight plasmids revealed the σ^{54} promoter sequence at one or other end of the insert. No insert was completely sequenced in one direction, the maximum number of bases read after the PCR primer being 251 (for Transformant #1).

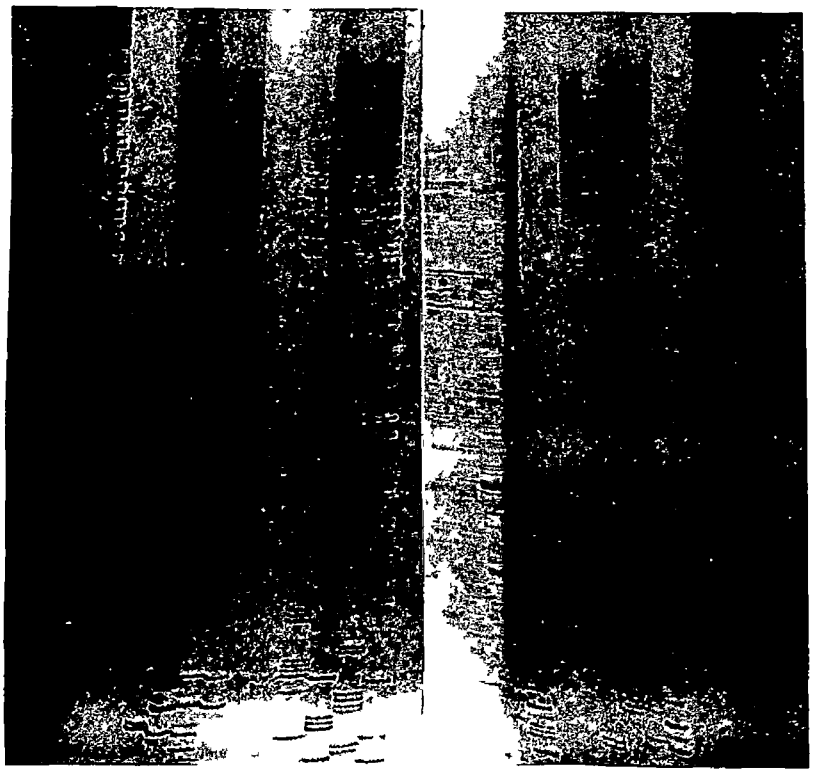


Plate 4.5 : An example of the upper portion of the autoradiogram obtained from a sequencing gel. Each set of four adjacent lanes contained A, C, G and T reactions respectively.
The order of the sets of reactions was as follows (left to right) :
#55 / M13 Universal primer - 3rd, 2nd and 1st loadings
#1 / SP6 promoter primer - 1st, 2nd and 3rd loadings

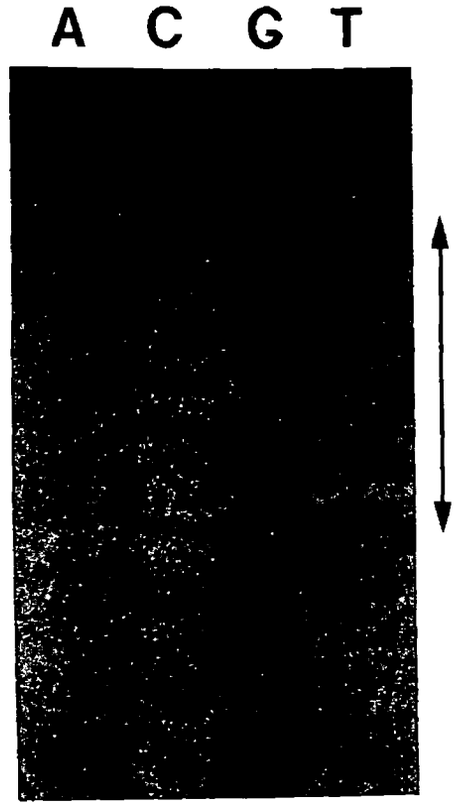


Plate 4.6 : An enlarged portion of the autoradiogram shown in Plate 4.5 showing the sequence of the initial σ^{54} consensus PCR primer. (Taken from the 3rd loading of sequencing reactions using transformant #1 and the SP6 promoter primer).

Table 4.6 shows the sequence data obtained when reading in from the "σ^{54} consensus primer" end of the insert. Reading from the T7/T3α primer end tended to give less information due to the number of bases of pUC18 DNA occurring before the insert was reached (i.e. between the T7/T3α region and the *Bam*HI site in the multiple cloning site). However a substantial portion was read for #55, and lesser amounts for #1, and for #6 (using the SP6 primer). (See Table 4.7.)

> transformant #6 (156 bp)
gtaaaacgac ggccag.....(95 bases).....**ctggc acgccttttg**
cacgtacagc cctggggccg catgctgttg cgcattcatg tgtccgaaca
accgaaatag cttaaacaac aaaggaagca agatggcagc tctgcgtcag
atcgcgttct acggtaaggg gggtatcggc aagtcacgac ctcccaaaat
acatcg

> transformant #30 (169 bp)
gtaaaacgac ggccag.....(95 bases).....**ctggc acgccttttg**
gcccggagtg aagctgtttt atttagcncn nctgcgcagg agttcacccc
tgaagaccgc tgcgtctgcc gtatgccgtg tactttgacc ggataggaag
gactattttc ctcgctgtgc tttacggtcg caaggccttg gctaatgtag
ttcgcagacg cgacgcgtg

> transformant #31 (174 bp)
gtaaaacgac ggccag.....(95 bases).....**ctggc acgccttttg**
cggtccttga gaaacggatc gctggaataa gcattcctga cttttgggcc
cttcatgccg tggctgagcg tgaagtggaa atcgcgctag tttctgaggc
gtgacgaact ccggctgccc gctgaatacc accgaaagaa ccgggcgtag
aggaacgtca catattcacc taca

> transformant #42 (177 bp)
gtaaaacgac ggccag.....(95 bases).....**ctggc acgccttttg**
ggcctctgac agaatgttga tcgcgaagtg ctcggctgcg gtctatgtcg
agtagtcgcg cgcagtcttg gcaacgcaaa cgagcagcag cgcgatccaa
ggatacagac gtaaaggagt tggcagtgaa accaaccggt ctggcacgcc
ttttggcccg gagtgaacgt gttttaa

> transformant #45 (112 bp)
gtaaaacgac ggccag.....(95 bases).....**ctggc acgccttttg**
gcccggagtg aagctgtttt atttagcaca gctgcgcagg agttcacccc
tgaagaccgc tgcgttctgc cgattccgta ctttgaccgg ataggaagga
ctatttttct ct

> transformant #54 (179 bp)
gtaaaacgac ggccag.....(95 bases).....**ctggc acgccttttg**
gtgtgtatgt gtatgtatgt gtttggttgc gggggcagga tttgaacctg
cnnccttcag gttatgagcc tgacgagcta ccgggcggct ccaccccgcg
ttatccgagt tttgcgaana aaatcttgnc cgagttttgc gaanaaaatc
tcgtctgagt tttgcgagcc aaaatctcg

Table 4.6 : (A) Partial sequences of transformants, σ^{54} end of insert; M13 Universal.

The above were sequenced using the M13 Universal primer, and are shown preceeded by the sequencing primer and starting with the σ^{54} consensus (PCR) primer (both primers in **bold**). 5' to 3' direction. The number of base pairs referred to in brackets indicates those after the PCR primer sequence.

> transformant #1 (251 bp)
atttaggtga cactata ...(80 bases)..... **ctggc acgcctttg**
ccgagctagg atcgtgtcga tcttgcgccg tgtccagcta tcgaatgtct
cgctggcccn nnncnccacc gcgacttcga tcgcctcnnc acgaagcnct
tccatcattt gtctggctga aatccgcacg acgaactgtg cccctgttgg
ccggatatcc accagtttcg atttgccgag gcgatgagcg cttcgnacgg
gttgacgcga cacgcccatg cgcgtggcat ttctgttcgg agagccgcat
t

> transformant #55 (114 bp after 2nd primer repeat)
atttaggtga cactata ...(80 bases)..... **ctggc acgcctttg**
cacgatcagc **tctggcacgc cttttg**gccc ggagtgaagc tgttttattt
agcacagctg cgcaggagtt caccccctgaa gaccgctgcg ctgtccgtat
gccgatgctt tgacctggat aggaaggact attttctcgc

Table 4.6 : (B) Partial sequences of transformants, σ^{54} end of insert; SP6.
The above were sequenced using the SP6 Promoter primer, and are shown preceeded by the sequencing primer and starting with the σ^{54} consensus (PCR) primer (both primers in **bold**). 5' to 3' direction.
(Note the repeat of the latter primer in #55.)

> transformant #1 (85 bp)
ggatcattgg aatcgggtgt agcgttctca ggccggcccg cncggtgcca
gccctccctg ttctaccggt ttnnngattt ctgttgng

> transformant #6 (88 bp)
ggatcactgt catctttctg agctctgcgt gctgaacgat attgtcnncnc
ggcacgaagt ggatagcgtt ggaattgagg cggcggcaag

> transformant #55 (170 bp)
ggatctcaaa aggcgtncca gatgctgtca tgtgagagca tgtcgaccca
tcttcggaag gagaagcgaa tngctgcaca gcagccnctc cgcgtctgcg
aatacattag ccaagcgcct tnnaccgtaa agcacagnag gaaaatagtc
cttcctatcc ggtcaagtac ggcatac

Table 4.7 : Sequence data read from T7/T3α primer end of insert. Data given starting from *Bam*HI/*Sau*3AI site (**bold**), in a 5' to 3' direction. Base pairs in brackets refer to number after the *Bam*HI/*Sau*3AI site.

4.1.7 Analysis of sequences

4.1.7.1 Homologies

All sequences obtained were submitted for homology searching against known sequences using the program BLAST, as detailed in Materials and Methods (Section 2.15.6).

Transformant #6 gave the most significant results, showing almost 100% identity to the *R. meliloti nifH* gene over the full length of its sequence. In total 58 sequences showing homology were found, mostly in the nitrogenase genes of various organisms. Table 4.8 shows an alignment of *R. meliloti nifH* and the sequence of transformant #6.

Transformant #54 was found to have a stretch of 77 bases with high homology to the Met-tRNA gene of a wide range of organisms, but on the minus strand. The first 20 of these sequences are listed in Table 4.9. There were also some sequences with positive homologies, listed in Table 4.10. Most of these are from choroplast DNA, and for the same region in the insert, and most have also been identified as coding for the complement of tRNA-Met. There are also three bacterial operons showing homology: a *Thermoplasma acidophilum* operon containing RNA polymerases and tRNA-met (complement), the *sla* gene of *Methanococcus voltae* (but the region of homology occurs after the end of the *sla* ORF) and the *hus5* gene of *Saccharomyces pombe*.

Transformant #31 showed two short regions of homology - one to the negative strand of the alpha-PS1 gene in *Drosophila melanogaster*, the other to part of an unidentified reading frame from *Proteus vulgaris*. However both regions were small and the probabilities of non-significant homology high (See Table 4.11).

The following showed no homology to any previously determined sequence: 1, 30, 42, 45 and 55.

Upper line: transformant #6

Lower line: *R.meliloti* nitrogen fixation gene, symbiotic promoter region P1. Length = 348 bp. Accession number: gb|K01468|RHMNIFPR3

Score = 639 (176.6 bits), Expect = 2.1e-54, Sum P(2) = 2.1e-54

Identities = 131/135 (97%), Positives = 131/135 (97%), Strand = Plus / Plus

```
1     CACGTACAGCCCTGGGGCCGCATGCTGTTGCGCATTCATGTGTCCGAACAACCGAAATAG 60
      |||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||
176   CACGATCAGCCCTGGGCGCGCATGCTGTTGCGCATTCATGTGTCCGAACAACCGAAATAG 235

61    CTTAAACAACAAAGGAAGCAAGATGGCAGCTCTGCGTCAGATCGCGTTCTACGGTAAGGG 120
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
236   CTTAAACAACAAAGGAAGCAAGATGGCAGCTCTGCGTCAGATCGCGTTCTACGGTAAGGG 295

121   GGGTATCGGCAAGTC 135
      |||||||||||||||
296   GGGTATCGGCAAGTC 310
```

Score = 95 (26.3 bits), Expect = 2.1e-54, Sum P(2) = 2.1e-54

Identities = 19/19 (100%), Positives = 19/19 (100%), Strand = Plus / Plus

```
135   CACGACCTCCCAAAATACA 153
      |||||||||||||||||||
311   CACGACCTCCCAAAATACA 329
```

Table 4.8 : Alignment of transformant #6 with *R. meliloti nifH*. (Bases 1 - 135 and 135 - 153).

Note : P = Poisson P value, and indicates the probability of the same number of matches occurring by chance alone. A smaller value for P indicates a greater statistical significance, whilst values approaching 1 are less significant.

Sequences producing High-scoring Segment Pairs:

Accession number	Description	High Probability Score	P(N)	Bases of #54	
gb|L10238|BAORTGAMET	*Bartonella bacilliformis* transfer RNA f-met	362	2.2e-24	100 - 24	
emb|X53854|RSRRNB	*R. sphaeroides* ribosomal RNA operon rrnB	377	9.4e-24	101 - 22	
emb|X53853|RSRRNA	*R. sphaeroides* ribosomal RNA operon rrnA	369	4.3e-23	101 - 20	
emb|X53855|RSRRNC	*R. sphaeroides* ribosomal RNA operon rrnC	367	6.4e-23	101 - 24	
gb|K00310|BACTRMF	*B. subtilis* initiator Met-tRNA-f.	326	2.2e-21	101 - 24	
emb|X60981|BSTRN	*Bacillus* sp. strain PS3 genes for tRNAs	345	4.2e-21	100 - 22	
gb|L08236|BACTGMD	*Bacillus subtilis* 23S rRNA, 5S rRNA, tRNA-Met,	344	4.5e-21	102 - 17	
gb|K01389|BACTGTRND	*B. subtilis* 168 trrnD gene cluster	337	1.9e-20	102 - 22	
gb|K01390|BACTGTRNE	*B. subtilis* 168 trrnE gene cluster	337	1.9e-20	102 - 22	
gb|K00637|BACRGRRNB	*B. subtilis* rrnB operon	337	2.0e-20	102 - 22	
gb|M21681|ECOTGMZY	*E. coli* Met-tRNA (metY and metZ) genes.	330	3.8e-20	101 - 17	*
dbj|D10260|TTHTRNA	*T. thermophilus* Met-tRNA.	317	4.7e-20	100 - 24	
gb|K00308|TTHTRMF1	*T. thermophilus* initiator Met-tRNA-f1.	317	4.7e-20	100 - 24	
gb|M25601|ECOTRFM	*E. coli* complete transfer RNA-fMet.	308	6.0e-19	100 - 24	
gb|M25600|ECOFMETTRN	*Escherichia coli* transfer RNA-fMet	301	3.3e-18	100 - 24	
gb|M21580|SYNADO47	Synthetic *E. coli* Ado-47 (tRNA-f-Met)	299	5.2e-18	100 - 24	
gb|M92071|STRTGMFA	*Streptococcus* sp. group B transfer RNAs	297	6.0e-18	105 - 27	
emb|X70449|ECTRNAM	*E. coli* gene for transfer RNA-Met	294	1.6e-17	100 - 24	
emb|X70452|ECTRNAM2	*E. coli* gene for transfer RNA-Met	285	1.2e-16	100 - 24	
gb|M21680|ECOTGM1Y	*E. coli* Met-tRNA (metY) gene.	299	2.1e-16	100 - 24	

Table 4.9 : The twenty highest scoring sequences showing homology to md54. Where part of an operon, the specific gene is that for tRNA-Met in all cases. Percentage homologies range from 83 - 96 %, but for the best matches there is only one actual mismatched base (and two N's).
* There were also two shorter regions of homology, within the same range (of md54), and also in a negative direction.

Accession number	Description	Score	P(N)	Bases (#54)
emb¦X01647¦CHMPTRN	*Marchantia polymorpha* chloroplast DNA	258	2.6e-12	20 - 104
sp¦X04465¦CHMPXX	Liverwort *M. polymorpha* chloroplast DNA	258	2.7e-12	20 - 104
sp¦Z00044¦CHNTXX	Tobacco chloroplast genome DNA.	244	4.8e-11	26 - 100
gb¦M81884¦EPFCPCG	*Epifagus virginiana* chloroplast DNA	239	1.3e-10	26 - 99
emb¦V00158¦CHEG01	Chloroplast *Euglena gracilis* tRNA genes	187	4.0e-06	26 - 103
emb¦X68198¦TARPOG	*T.acidophilum rpoH, rpoB, rpoA12, trnM*	185	6.7e-06	26 - 122
gb¦L02941¦MZETRNA	*Zea mays* chloroplast transfer RNAs	184	7.3e-06	28 - 99
emb¦X02560¦CHTATRN1	Wheat chloroplast genes for tRNAs	184	7.9e-06	28 - 99
sp¦X15901¦CHOSXX	Rice complete chloroplast genome	184	8.2e-06	28 - 99
gb¦M68929¦MPOMTCG	*Marchantia polymorpha* mitochondrion DNA	183	1.0e-05	22 - 81
gb¦M59200¦MVOATPASE	*Methanococcus voltae sla* gene	172	8.5e-05	24 - 80
emb¦X81846¦SPHUS5	*Saccharomyces pombe hus5* gene	147	0.012	24 - 75

Table 4.10 : Sequences showing positive homology to #54

---

emb|X73975|DMAPS1 *D.melanogaster alpha-PS1* (gene for integrin)
Homology to minus strand, 63 bp after end of ORF.
P = 0.35, Identities = 37/51 (72%).
#31· bases 85 - 35, DMAPS1 bases 3846 - 3896

```
#31          GCGATTTCCACTTCACGCTCAGCCACGGCATGAAGGGCCCAAAAGTCAGGA
             |||| ||||  ||  |||||||||||| ||||   |||  |||| |  |||
DMAPS1       GCGACTTCCTGTTACCGCTCAGCCACGACATGGTTGGCGAAAAACTACGGA
```

>emb|X06151|PVORF1 *Proteus vulgaris orf* operon (unidentified). homology inside *orfC*, to positive strand
P = 1.0, Identities = 29/37 (78%)
#31 bases 63 - 99, PVORF1 bases 978 - 1014

```
#31          GCTGAGCGTGAAGTGGAAATCGCGCTAGTTTCTGAGG
             | | |||||||||||| | |||||  |||||| ||| |
PVORF1       GTTAAGCGTGAAGTGAATATCGCATTAGTTTGTGAAG
```

[The coding region *orfC* is from base 900 - 1190 There is a gg/gc motif starting at base 891, but otherwise it is a bad match for the NtrA promoter sequence (reading gggttgttatgtgc)]

Table 4.11 : The two regions of homology found for transformant #31 giving their GenBank accession numbers, the regions of homology and notes on these regions relative to open reading frames etc.

4.1.7.2 Open reading frames

The "map" option of GCG:The Wisconsin Package was used to find open reading frames in the sequences determined. These are shown for transformants 1, 6, 30, 31, 42 and 54 in Table 4.12.

Transformant #1

```
  CCGAGCTAGGATCGTGTCGATCTTGCGCCGTGTCCAGCTATCGAATGTCTCGCTGGCCCN
1 ---------+---------+---------+---------+---------+---------+ 60
a P  S  *                                                     -
b  R  A  R  I  V  S  I  L  R  R  V  Q  L  S  N  V  S  L  A  ? -
c   E  L  G  S  C  R  S  C  A  V  S  S  Y  R  M  S  R  W  P  ?-

   NNNCNCCACCGCGACTTCGATCGCCTCNNCACGAAGCNCTTCCATCATTTGTCTGGCTGA
61 ---------+---------+---------+---------+---------+---------+ 120
a                                                             -
b    ?  ?  T  A  T  S  I  A  S  ?  R  S  ?  S  I  I  C  L  A  E -
c     ?  P  P  R  L  R  S  P  ?  H  E  A  L  P  S  F  V  W  L  K-

    AATCCGCACGACGAACTGTGCCCCTGTTGGCCGGATATCCACCAGTTTCGATTTGCCGAG
121 ---------+---------+---------+---------+---------+---------+ 180
a                                                             -
b    I  R  T  T  N  C  A  P  V  G  R  I  S  T  S  F  D  L  P  R -
c     S  A  R  R  T  V  P  L  L  A  G  Y  P  P  V  S  I  C  R  G-

    GCGATGAGCGCTTCGNACGGGTTGACGCGACACGCCCATGCGCGTGGCATTTCTGTTCGG
181 ---------+---------+---------+---------+---------+---------+ 240
a      M  S  A  S  ?  G  L  T  R  H  A  H  A  R  G  I  S  V  R  -
b    R  *                              M  R  V  A  F  L  F  G  -
c     D  E  R  F  ?  R  V  D  A  T  R  P  C  A  W  H  F  C  S  E-

    AGAGCCGCATT
241 ---------+- 251
a   R  A  A    -
b    E  P  H   -
c     S  R  I  -
```

Table 4.12 : (A). Possible open reading frames in inserts sequenced; Transformant #1.
[*Sau*3AI sites are in **bold**, and protein sequences for the three forward directions are shown.]

Transformant #6

```
  CACGTACAGCCCTGGGGCCGCATGCTGTTGCGCATTCATGTGTCCGAACAACCGAAATAG
1 ---------+---------+---------+---------+---------+---------+ 60
a H  V  Q  P  W  G  R  M  L  L  R  I  H  V  S  E  Q  P  K  *   -
b  T  Y  S  P  G  A  A  C  C  C  A  F  M  C  P  N  N  R  N  S  -
c   R  T  A  L  G  P  H  A  V  A  H  S  C  V  R  T  T  E  I  A -

   CTTAAACAACAAAGGAAGCAAGATGGCAGCTCTGCGTCAGATCGCGTTCTACGGTAAGGG
61 ---------+---------+---------+---------+---------+---------+ 120
a                                                               -
b   L  N  N  K  G  S  K  M  A  A  L  R  Q  I  A  F  Y  G  K  G  -
c    *                                                          -

    GGGTATCGGCAAGTCACGACCTCCCAAAATACATCG
121 ---------+---------+---------+------ 156
a                                       -
b    G  I  G  K  S  R  P  P  K  I  H    -
c                                       -
```

Transformant #30

```
  GCCCGGAGTGAAGCTGTTTTATTTAGCNCNNCTGCGCAGGAGTTCACCCCTGAAGACCGC
1 ---------+---------+---------+---------+---------+---------+ 60
a A  R  S  E  A  V  L  F  S  ?  ?  A  Q  E  F  T  P  E  D  R   -
b  P  G  V  K  L  F  Y  L  A  ?  L  R  R  S  S  P  L  K  T  A  -
c   P  E  *                                                     -

   TGCGTCTGCCGTATGCCGTGTACTTTGACCGGATAGGAAGGACTATTTTCCTCGCTGTGC
61 ---------+---------+---------+---------+---------+---------+ 120
a  C  V  C  R  M  P  C  T  L  T  G  *                          -
b   A  S  A  V  C  R  V  L  *                                  -
c                                                              -

    TTTACGGTCGCAAGGCCTTGGCTAATGTAGTTCGCAGACGCGACGCGTG
121 ---------+---------+---------+---------+--------- 169
a                        M  *                        -
b                                                    -
c                                                    -
```

Table 4.12 : (B). Possible open reading frames for transformants #6 and #30.

Transformant #31

```
   CGGTCCTTGAGAAACGGATCGCTGGAATAAGCATTCCTGACTTTTGGGCCCTTCATGCCG
1 ---------+---------+---------+---------+---------+---------+ 60
a R  S  L  R  N  G  S  L  E  *                       M  P   -
b  G  P  *                                                   -
c   V  L  E  K  R  I  A  G  I  S  I  P  D  F  W  A  L  H  A  V -

   TGGCTGAGCGTGAAGTGGAAATCGCGCTAGTTTCTGAGGCGTGACGAACTCCGGCTGCCC
61 ---------+---------+---------+---------+---------+---------+ 120
a  W  L  S  V  K  W  K  S  R  *                              -
b                                                             -
c    A  E  R  E  V  E  I  A  L  V  S  E  A  *                 -

    GCTGAATACCACCGAAAGAACCGGGCGTAGAGGAACGTCACATATTCACCTACA
121 ---------+---------+---------+---------+---------+---- 174
a                                                             -
b                                                             -
c                                                             -
```

Transformant #42

```
   GGCCTCTGACAGAATGTTGATCGCGAAGTGCTCGGCTGCGGTCTATGTCGAGTAGTCGCG
1 ---------+---------+---------+---------+---------+---------+ 60
a G  L  *                                                     -
b  A  S  D  R  M  L  I  A  K  C  S  A  A  V  Y  V  E  *       -
c   P  L  T  E  C  *                          M  S  S  S  R  A -

   CGCAGTCTTGGCAACGCAAACGAGCAGCAGCGCGATCCAAGGATACAGACGTAAAGGAGT
61 ---------+---------+---------+---------+---------+---------+ 120
a                                                             -
b                                                             -
c    Q  S  W  Q  R  K  R  A  A  A  R  S  K  D  T  D  V  K  E  L -

    TGGCAGTGAAACCAACCGGTCTGGCACGCCTTTTGGCCCGGAGTGAACGTGTTTTAA
121 ---------+---------+---------+---------+---------+------- 177
a                                                             -
b                                                             -
c     A  V  K  P  T  G  L  A  R  L  L  A  R  S  E  R  V  L   -
```

Table 4.12 : (C). Possible open reading frames for transformants #31 and #42.

Transformant #54

```
  GTGTGTATGTGTATGTATGTGTTTGGTTGCGGGGGCAGGATTTGAACCTGCNNCCTTCAG
1 ---------+---------+---------+---------+---------+---------+ 60
a V  C  M  C  M  Y  V  F  G  C  G  G  R  I  *                    -
b  C  V  C  V  C  M  C  L  V  A  G  A  G  F  E  P  A  ?  F  R  -
c   V  Y  V  Y  V  C  V  W  L  R  G  Q  D  L  N  L  ?  P  S  G -

   GTTATGAGCCTGACGAGCTACCGGGCGGCTCCACCCCGCGTTATCCGAGTTTTGCGAANA
61 ---------+---------+---------+---------+---------+---------+ 120
a     M  S  L  T  S  Y  R  A  A  P  P  R  V  I  R  V  L  R  ?   -
b   L  *                                                        -
c    Y  E  P  D  E  L  P  G  G  S  T  P  R  Y  P  S  F  A  ?  K -

    AAATCTTGNCCGAGTTTTGCGAANAAAATCTCGTCTGAGTTTTGCGAGCCAAAATCTCG
121 ---------+---------+---------+---------+---------+--------- 179
a   K  S  ?  P  S  F  A  ?  K  I  S  S  E  F  C  E  P  K  S     -
b                                                               -
c     I  L  ?  E  F  C  E  ?  N  L  V  *                       -
```

Table 4.12 : (D). Possible open reading frames in transformant #54.

4.2 Discussion

4.2.1 The PCR reaction

The PCR reaction used in this work showed itself to be extremely variable and difficult to reproduce. The annealing temperature was lowered in order to reduce the specificity of the reaction, but the effects of this strategy were by no means uniform, i.e. comparing the number of bands obtained at 38, 36 and 35°C (Section 4.1.3.4). In the PCR reaction shown in Plate 4.2, the concentration of magnesium chloride was also varied, as a higher concentration should lower specificity.
This appears to be the case for reactions 3 and 4 (i.e. more bands for the higher $MgCl_2$ concentration) but does not follow the same pattern for reactions 7 and 8, where the latter shows no discernible bands despite having the higher concentration.
However it is possible that some of this variability may be due to variation in the template. Unfortunately, due to the nature of the cloning, the gene bank had to be transformed before it could be used as a template for PCR. This would have led to enrichment for some constructs and loss of others, and possibly gave rise to different sample aliquots containing different portions of the overall gene bank rather than a representative sample.

No reactions were found to work with the M13 Universal primer. This may be related to the fact that its annealing temperature (47°C) was further removed from that of the σ^{54} consensus primer (43°C) than was that of the T7/T3α primer (41°C).

4.2.2 Sequence results

4.2.2.1 Identification of a known σ^{54}-dependent gene

The sequence obtained for the insert in transformant #6 is undoubtedly that of the *nifH* gene of *R. meliloti*. This gene is known to be transcribed by σ^{54}-RNA polymerase (with the activator NifA) and σ^{54} has been shown to bind specifically to a promoter region (e.g. Cannon *et al.* 1993) differing only in one base from the sequence of the PCR primer used (See Table 4.2). The isolation of this sequence shows that the PCR reaction amplified the type of fragment predicted, despite a one base difference between the primer chosen (the "σ^{54} consensus " primer) and the actual primer sequence described for this gene. Hence it acts as an internal positive control on this strategy for the isolation of σ^{54}-dependent promoters.

4.2.2.2 Isolation of tRNA-met

Transformant #54 appears to contain the sequence for the transfer RNA specific for methionine. (The possible ORF mentioned above starts within this region of homology.) The fact that it is in a negative orientation relative to the σ^{54} consensus primer rules out σ^{54}-regulated transcription of the actual transfer RNA, although there are no apparent promoter sites from the other direction either (σ^{54} or σ^{70}). However, tRNA-met is often part of a larger operon (e.g. in *Bacillus* sp. PS3 [Fink *et al.* 1991] and in *B. subtilis* [Rudner *et al.* 1993]), and is often transcribed along with other tRNAs and rRNA. The remainder of #54 shows no homology to any other known sequence. On closer examination, the region following the complement of tRNA-met consists of three almost exact repeats of a stretch of 25 bases. The significance of this is unclear, unless these are possibly weak termination regions for another ORF reading into this from the opposite direction (i.e. in the same orientation as "correct" tRNA-met). It is

possible that what was amplified is indeed the gene for tRNA-met, and that the opposite strand of the DNA downstream from it contains a region with some homology to the σ^{54} consensus primer, but not an actual promoter region (a pseudo σ^{54}-recognition site). If this is indeed the case, the question arises as to whether this pseudo-site is capable of binding the σ^{54} subunit, as the sigma factor appears to remain bound to its recognition sites even when not involved in transcription (e.g. because of the unavailability of phosphorylated activator protein; see Section 1.4.4.4). The presence of pseudo-sites in the genome also capable of binding the sigma factor would appear to be wasteful for the cell. However the homology needed to bind σ^{54} *in vivo* and the homology required to yield a product in the PCR reaction employed in this work, are not necessarily the same. In PCR, the 3' end of the primer is most important, while for recognition and binding of σ^{54}, certain sequence elements (especially the GG/GC doublets) are clearly more important than others, as shown by the degree to which they are conserved between genes and even between species.

The possibility must also be considered that σ^{54} does transcribe a product that is the complement of tRNA-met, i.e. an antisense tRNA. The hypothetical effect of such a transcript would probably be a very strong repression of protein synthesis through its interaction with initiator tRNA-met. This would appear to be a very extreme and drastic step for any cell to take and it is doubtful that there would ever be circumstances that required it. However it must be bourn in mind that the rhizobia do undergo drastic changes in their metabolism when they become bacteroids in the cytoplasm of plant root-nodule cells. Certainly protein synthesis accompanies this change - particularly synthesis of the nitrogenase complex, a process requiring σ^{54} and the NifA activator protein - but it is possible that after this change has taken place, the rate of protein synthesis in the bacteroid is greatly reduced.

Another possibility for σ^{54}-mediated transcription of this region is that the transcription does not end after the tRNA complement, but continues through the repeat regions and into another unknown ORF. Regions of mRNA homologous to tRNAs have been previously found (e.g. homology to tRNA-his at the start of the *his* operon of *E. coli*) and implicated in control of the operon (Ames *et al.* 1983). The complement homology found here may be an additional control factor in a gene also under σ^{54} control. It is interesting that one of the homologues found to #54, the *T. acidophilum* tRNA-met gene, occurs in a minus orientation directly before an unknown ORF (Klenk *et al.*, 1992), but no specific purpose for this has been noted.

4.2.2.3 Other transformants sequenced

The other isolates showing no homology to known sequences (including #31 where the homology found was very low) may also be previously unknown genes under σ^{54} control, but further work would be necessary to verify this.

4.2.2.4 Open reading frames

Apart from #6, transformants #1, #42, and #54 also show possible open reading frames (Table 4.12 A, B, C and D). There are three possible ORFs starting in #1, at bases 45, 185 and 218 respectively. There appears to be no set number of bases between the transcription and translation start site for σ^{54}-regulated genes, but for the examples given in Table 4.2, this varies from 26 - 116 bp. This would make the ORF starting at base 45 the most likely to be of significance. This is also the distance from the (putative) promoter to the ORF in #42, while #54 has one starting at base 64. Transformants #s 30, 31, 45 and 55 show no ORFs of any significant length but of course a single base insertion or deletion - either physically in the amplification, or through error in reading the sequencing

gel - would be enough to disrupt or obscure an existing one. (Note the apparent one base deletion in #6 relative to previously sequenced *nifH* Table 4.11). *Sau3AI* sites also are possible sites of ligation between unrelated fragments of genomic DNA during the creation of the gene bank. It is also possible that an ORF may not occur until further downstream from the promoter than has been sequenced here.

4.2.2.5 Homologies between transformants

The sequences of numbers 30 and 45 appear to be homologous. So also does #55, but after first reading 10 bases similar to the *nifH* sequence, then repeating the σ^{54} consensus primer. Table 4.13 shows an alignment of these three sequences. It is possible that mistakes in the PCR reaction have led to the differences between the three. However it is also possible that there is more than one copy of the gene in question to be found on the genome, and that differences already existed before the creation of the gene bank and the PCR reaction.

Alignment of 30, 45, and 55

```
30     gcccggagtgaagctgttttatttagcncnnctgcgcaggagttcacccctgaagaccgctg
45     gcccggagtgaagctgttttatttagcacagctgcgcaggagttcacccctgaagaccgctg
55    ·gcccggagtgaagctgttttatttagcacagctgcgcaggagttcacccctgaagaccgctg

30     cgtctgccgtatgccgtgtactttgacc ggataggaaggactattttcctcgctgtgctt
45    :        cgattcc  gtactttgacc ggataggaaggactatttttctctcgttctgc
55(f):         gtatgccg atgctttgacctggataggaaggactatttt ctcgccgctgtcc
55(r):         gtatgccg  tact tgacc ggataggaaggactattttcct nctgtgctt

30     tacggtcgcaaggc cttggctaatgtagttcgcagacgcgacgcg  tg
55(f) tacggtnn aaggcgcttggctaatgta ttcgcagacgcggagnggctg
55(r) ctgtgcagcnattcgcttctccttccgaagatgggtcgacatgctctcacatgacagcatc

55(r):tggnacgccttttgatc
```

Table 4.13 : An alignment of sequences for transformants #30, #45 and #55, also including #55 read from the opposite side of the insert. (f = forward, i.e. from the σ^{54} Consensus primer end; r = reverse, from the T7/T3α primer end.)

4.2.3 Conclusion

The methodology described in this chapter was successful in isolating a gene under the control of σ^{54} on the basis of its having the specific promoter sequence associated with known examples of such genes. Only one of the known examples was among the eight transformants sequenced, but this does not rule out other known genes being among the pool of unsequenced transformants obtained. Selection of transformants for sequencing was on the basis of differences in size of insert only, but there is nothing to imply that the number of different sized PCR products equals the number of σ^{54} promoter sequences on the *R. meliloti* genome. Whether or not the gene bank was fully representitive (if enrichment took place during transformation and loss of certain constructs occurred), there is also the possibility of PCR products of roughly the same size containing different sequences.

An interesting question regarding these results is whether all the sequences obtained are actually regions transcribed by σ^{54}, or simply happen to show sufficient homology to its promoter sequence to allow the PCR reaction to take place. It is likely that at least some are sequences of true σ^{54}-dependent genes and as such would be interesting to sequence further and study in more detail.

Chapter 5 :

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Appendix A:

Complete BLAST results for transformant #6

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gb|K01468|RHMNIFPR3	R.melilоti nitrogen fixation gene, s...	639	2.1e-54	2
gb|J01781|RHMNIFH	r.melilоti nitrogenase reductase (ni...	580	8.2e-45	2
emb|V01215|RMNIFH	R. melilоti nitrogenase reductase st...	580	8.2e-45	2
gb|M23347|ALFMOS	Medicago sativa mos gene (required f...	594	3.1e-44	1
gb|L17073|RHMNIFANTR	Rhizobium melilоti NifA/NtrA consens...	585	4.4e-43	1
gb|L17071|RHMMOSA	Rhizobium melilоti rhizopine biosynt...	594	8.2e-42	1
gb|K01467|RHMNIFPR2	R.melilоti nitrogen fixation gene, s...	403	2.6e-26	1
gb|J01782|RHMNIFHDK	r.melilоti nitrogenase operon (nifhd...	389	5.8e-26	1
gb|I06891|I06891	Sequence 5 from patent EP 0339830.	335	3.9e-22	1
emb|X66124|RMPLNFE	R.melilоti plasmid DNA for nfe locus	324	2.4e-19	1
gb|L16502|RHMKR	Rhizobium sp. DNA sequence.	226	2.9e-14	2
gb|L16503|RHMQB	Rhizobium fredii DNA sequence.	258	1.1e-12	1
gb|I01719|I01719	Sequence 2 from patent US 4803165.	253	3.7e-12	1
gb|M26961|RHMNIFKDH3	Rhizobium sp. nitrogenase structural...	253	3.7e-12	1
emb|X51750|RLPNIF	Rhizobium leguminosarum putative nif...	211	1.6e-08	1
gb|M33774|RSPNIFHD	R.rubrum dinitrogenase reductase (ni...	211	2.4e-08	1
emb|X02479|RTRS2	R.trifolii (strain 843) repeated seq...	207	4.9e-08	1
emb|X02478|RTRS1	Rhizobium trifolii (strain 843) repe...	207	5.0e-08	1
gb|K00490|RHMNIFHY	R.trifolii nitrogenase genes nifh (c...	207	5.6e-08	1
gb|I04063|I04063	Sequence 3 from patent EP 0130047.	207	5.6e-08	1
gb|L11085|RHMNIFH1X	Rhizobium leguminosarum Fe protein (...	205	6.9e-08	1
gb|L11086|RHMNIFH2X	Rhizobium leguminosarum Fe protein (...	205	6.9e-08	1
gb|L11087|RHMNIFH3X	Rhizobium leguminosarum Fe protein (...	205	6.9e-08	1
gb|M10587|RHMNIFHAZ	R.phaseoli plasmid p42-D nitrogenase...	205	8.1e-08	1
gb|M15942|RHMNIFHCZ	R.phaseoli plasmid p42-D nitrogenase...	205	8.1e-08	1
gb|M36435|RHMNIFHAA	R.leguminosarum nitrogen fixation pr...	202	1.3e-07	1
gb|M15401|RHMNIFHX	Cowpea Rhizobium IRc78 nifH gene enc...	196	3.7e-07	1
gb|K02676|RHMNIFHZ	Rhizobium BTAi1 nifH gene, promoter ...	196	4.0e-07	1

Sequences producing High-scoring Segment Pairs:		Score	P(N)	N
gb|M29401|RCANIFPRB	R.capsulata nifH gene, 5' end.	176	4.3e-07	1
emb|X17522|FANIFH	Frankia (HRN18a) DNA for nitrogenase...	195	6.2e-07	1
emb|X51609|ABNIFHGE	A.brasilense DNA for nifH, the gene ...	194	7.9e-07	1
emb|X51500|ABNIFH	Azospirillum brasilense NifH gene	194	7.9e-07	1
gb|S44003|S44003	nif gene cluster: nifH=nitrogeanse F...	194	8.2e-07	1
gb|M64344|AZSFEFEMO	A.brasilense Fe protein and FeMo pro...	194	8.2e-07	1
gb|M29402|RCANIFPRC	R.capsulata nifH gene, 5' end.	176	1.1e-06	1
gb|K00487|RHPNIFH	Parasponia rhizobium nitrogenase (ni...	187	3.3e-06	1
emb|X73983|FSNIF	Frankia sp. nif-H gene for nitrogena...	179	1.6e-05	1
gb|M29400|RCANIFPRA	R.capsulata nifHDK operon, promoter ...	177	1.9e-05	1
gb|K01620|RHMNIFHW	Rhizobium japonicum nifH gene encodi...	178	1.9e-05	1
gb|M15270|RCANIFHD	Rhodobacter capsulatus nifH gene enc...	177	2.5e-05	1
emb|X07866|RCNIFH	Rhodobacter capsulatus nifH gene	176	2.8e-05	1
gb|M16710|RHMNIFH2	Rhizobium nifH2 gene encoding nitrog...	176	2.9e-05	1
gb|M16709|RHMNIFH1	Rhizobium nifH1 gene encoding nitrog...	176	2.9e-05	1
emb|X63352|RCFDXD	R.capsulatus fdxD, nifH and nifD (5'...	176	3.0e-05	1
gb|M29367|RHMNODD2A	R.meliloti nodulation protein (nodD2...	163	0.00039	1
emb|X03916|ACNIFH	Azotobacter chroococcum MCD 1 nifH* ...	151	0.0043	1
emb|X51756|ACVNF	Azotobacter chroococcum vnfH, D, G, ...	151	0.0045	1
gb|L04499|ANANIFHDU	Anabaena sp. dinitrogenase reductase...	146	0.012	1
emb|V00001|A7NIFH	Anabaena 7120 nifH gene (nitrogenase...	137	0.067	1
emb|Z31716|NPNIFHDU	Nostoc PCC 6720 nifH, nifD, nifU genes	137	0.069	1
emb|V01482|A7NIFX	Anabaena 7120 genes nifH and nifD. n...	137	0.069	1
dbj|D00666|PEENIFUHD	P.boryanum nifU gene for NifU protei...	137	0.069	1
gb|L23514|NOSMOFENIF	Nostoc commune (UTEX 584) nitrogen f...	137	0.069	1
gb|J05111|ANANIFBH	Anabaena sp. (clone AnH20.1) nitroge...	137	0.070	1
gb|M36688|AVINIFH2A	A.vinelandii nitrogen fixation prote...	136	0.082	1
emb|X13519|AVNIFH2	A.vinelandii ORF and nifH2 gene for ...	136	0.082	1
gb|M32371|AVIVNFDG	A.vinelandii nitrogenase (vnfHDGK) g...	136	0.085	1
emb|X70033|RCANF	R.capsulatus anfH, anfD, anfG and an...	124	0.61	1

Appendix B:

Complete BLAST results for transformant #54

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gb|L10238|BAORTGAMET	Bartonella bacilliformis transfer RN...	362	2.2e-24	1
emb|X53854|RSRRNB	R. sphaeroides ribosomal RNA operon ...	377	9.4e-24	1
emb|X53853|RSRRNA	R. sphaeroides ribosomal RNA operon ...	369	4.3e-23	1
emb|X53855|RSRRNC	R. sphaeroides ribosomal RNA operon ...	367	6.4e-23	1
gb|K00310|BACTRMF	B.subtilis initiator Met-tRNA-f.	326	2.2e-21	1
emb|X60981|BSTRN	Bacillus sp. strain PS3 genes for tR...	345	4.2e-21	1
gb|L08236|BACTGMD	Bacillus subtilis 23S ribosomal RNA ...	344	4.5e-21	1
gb|K01389|BACTGTRND	B.subtilis 168 trrnD gene cluster co...	337	1.9e-20	1
gb|K01390|BACTGTRNE	B.subtilis 168 trrnE gene cluster co...	337	1.9e-20	1
gb|K00637|BACRGRRNB	B.subtilis rrnB operon with 23S rRNA...	337	2.0e-20	1
gb|M21681|ECOTGMZY	E.coli Met-tRNA (metY and metZ) genes.	330	3.8e-20	1
dbj|D10260|TTHTRNA	T. thermophilus Met-tRNA. >gb|K00309...	317	4.7e-20	1
gb|K00308|TTHTRMF1	T.thermophilus initiator Met-tRNA-f1.	317	4.7e-20	1
gb|M25601|ECOTRFM	E.coli complete transfer RNA-fMet. >...	308	6.0e-19	1
gb|M25600|ECOFMETTRN	Escherichia coli transfer RNA-fMet (...	301	3.3e-18	1
gb|M21580|SYNADO47	Synthetic E.coli Ado-47-containing t...	299	5.2e-18	1
gb|M92071|STRTGMFA	Streptococcus sp. group B transfer R...	297	6.0e-18	1
emb|X70449|ECTRNAM	E.coli gene for transfer RNA-Met	294	1.6e-17	1
emb|X70452|ECTRNAM2	E.coli gene for transfer RNA-Met	285	1.2e-16	1
gb|M21680|ECOTGM1Y	E.coli Met-tRNA (metY) gene.	299	2.1e-16	1
gb|K00311|ANITRMF	Anacystis nidulans initiator Met-tRN...	281	2.8e-16	1
emb|X04543|SGTRNM	Streptomyces griseus initiator trans...	281	2.8e-16	1
gb|M28401|ECOTGMETY	E.coli Met-tRNA-f2 (metY) gene, 5' end.	299	3.2e-16	1
gb|M92070|STRTGMFC	Streptococcus pyogenes transfer RNA-...	278	3.3e-16	1
emb|X00513|ECNUSA2	E.coli nusA operon including genes f...	299	3.9e-16	1
gb|U18997|ECOUW67	Escherichia coli K-12 chromosomal re...	299	4.0e-16	1
gb|M92072|STRTGMFB	Streptococcus sp. group G transfer R...	276	5.2e-16	1
gb|M32255|STMTGFMB	S.rimosus initiator tRNA gene.	271	1.8e-15	1
emb|X00576|MSTRNMET	Mycobacterium smegmatis initiator tr...	272	1.9e-15	1

Sequences producing High-scoring Segment Pairs:		Score	P(N)	N
emb|X61068|ALTRNA11	A.laidlawii 23S rRNA, 5S rRNA, tRNA-...	286	6.6e-15	1
gb|M32254|STMTGFMA	S.rimosus initiator tRNA gene.	262	5.1e-14	1
gb|K00320|SPICPTRMF	Spinach chloroplast initiator Met-tR...	254	8.1e-14	1
gb|M16880|PHVMTTRMF	Bean (P.vulgaris) mitochondrial fMet...	249	2.2e-13	1
gb|K00313|SOBCPTRMF	S.obliquus chloroplast initiator Met...	245	5.1e-13	1
gb|L11530|STATRNAA	Staphylococcus aureus transfer RNA s...	263	9.2e-13	1
gb|L15234|MYCTGC	Mycoplasma pneumoniae Cys-tRNA, Pro-...	261	1.2e-12	1
emb|X01647|CHMPTRN	Marchantia polymorpha chloroplast DN...	258	2.6e-12	1
sp|X04465|CHMPXX	Liverwort Marchantia polymorpha chlo...	258	2.7e-12	1
emb|X13879|CHCRPSBC	Chlamydomonas rheinhardtii chloropla...	257	3.2e-12	1
dbj|D00547|MYC9TRGC	M. capricolum tRNA gene cluster enco...	232	6.9e-12	2
emb|X04377|MILATRNM	Lupine mitochondrial gene for initia...	251	9.3e-12	1
gb|M20961|MPOCPTRMB	Liverwort chloroplast fMet-tRNA-cat.	230	1.1e-11	1
gb|K00319|PHVCPTRMF	Phaseolus vulgaris (bean) chloroplas...	227	2.0e-11	1
gb|K00312|MYCTRMF	Mycoplasma mycoides sp. capri initia...	227	2.0e-11	1
emb|X16759|MCTRFM	Mycoplasma capricolum transfer RNA-f...	227	2.0e-11	1
emb|X01017|CHNT10	Tobacco chloroplast genes for tRNA-f...	244	3.7e-11	1
sp|Z00044|CHNTXX	Tobacco chloroplast genome DNA.	244	4.8e-11	1
emb|X03715|SMTRNA1	Spiroplasma melliferum tRNA gene cluster	241	8.1e-11	1
emb|X61798|CHEVRPS	E.virginiana plastid rps14, rps2 and...	239	1.3e-10	1
gb|M81884|EPFCPCG	Epifagus virginiana chloroplast comp...	239	1.3e-10	1
emb|X03154|MMTRNA2	Mycoplasma mycoides transfer RNA gen...	232	4.9e-10	1
emb|X04131|CHSOPSA	Spinach plastid genes psaA, psaB and...	229	9.9e-10	1
emb|X05394|CHPSRP14	Pea chloroplast genes rps14, trnfM a...	223	3.0e-09	1
emb|X66090|SHTASP	S.hominis tRNA-Met & tRNA-Asp genes	182	4.0e-09	2
emb|Z12626|MIBSCCMS	Brassica sp. cybrid mitochondrial DN...	220	5.9e-09	1
emb|Z18896|MIRSORFBB	R.sativus mitochondrion orf138, orfB...	220	6.0e-09	1
gb|U10482|SSU10482	Synechocystis sp. putative endonucle...	145	1.1e-08	2
gb|U02970|PWU02970	Prototheca wickerhamii 263-11 comple...	216	1.4e-08	1
dbj|D17510|PINCPTRPG	Complete sequence of black pine (Pin...	216	1.4e-08	1
emb|X75364|LLTRRRM	L.lactis genes for tRNAs and 5S ribo...	182	2.8e-08	2

Sequences producing High-scoring Segment Pairs:		Score	P(N)	N
emb|X01221|TATRNMET	Thermoplasma acidophilum initiator t...	179	2.9e-07	1
emb|Z12627|MIBNNCO27	B.napus cybrids mitochondrial atpA g...	193	1.3e-06	1
emb|V00158|CHEG01	Chloroplast Euglena gracilis genes c...	187	4.0e-06	1
emb|Z11874|CHEGZ	Euglena gracilis Z Chloroplast DNA	187	4.5e-06	1
emb|X70810|CLEGCGA	E.gracilis chloroplast complete geno...	187	4.5e-06	1
emb|X68198|TARPOG	T.acidophilum genes rpoH, rpoB, rpoA...	185	6.7e-06	1
gb|L02941|MZETRNA	Zea mays chloroplast transfer RNA-Gl...	184	7.3e-06	1
emb|X02560|CHTATRN1	Wheat chloroplast genes for tRNA-Gly...	184	7.9e-06	1
sp|X15901|CHOSXX	Rice complete chloroplast genome	184	8.2e-06	1
gb|M68929|MPOMTCG	Marchantia polymorpha mitochondrion,...	183	1.0e-05	1
emb|X69095|SPTRNAMI	S.pombe tRNA-Met(i)	156	2.8e-05	1
gb|M59200|MVOATPASE	Methanococcus voltae S-layer structu...	172	8.5e-05	1
emb|X55109|MLTRNAP2	M. luteus gene for tRNA-Pro (CGG)	168	0.00011	1
gb|M65195|MSGTGGP	Mycobacterium smegmatis Pro- and Gly...	168	0.00014	1
emb|X69476|CPGSEA	C.psittaci gseA gene for 3-deoxy-D-2...	163	0.00051	1
emb|Z22659|CTGSEAF	C.trachomatis gseA gene	161	0.00076	1
gb|M64618|CHTKDOT	Chlamydia trachomatis 3-deoxy-d-mann...	161	0.00076	1
emb|X80061|CPKDOTR	C.psittaci gene for KDO-transferase	161	0.00076	1
emb|X01223|SATRNMET	Sulfolobus acidocaldarius initiator ...	141	0.0012	1
emb|X66152|ECTRNAMEA	E.coli tRNA-Met gene, complete CDS	137	0.0040	1
emb|Z31593|CPKDOTRA	C.pneumoniae (TW183) DNA for KDO tra...	150	0.0065	1
sp|X55522|MTPSAM2AT	M.tuberculosis pSAM2 attB-like sequence	143	0.0073	1
gb|M10370|YSPTGSM2	Yeast (S.pombe) Ser-tRNA (SUP12-UGA)...	147	0.0083	1
gb|M10354|YSPTGSM1	Yeast (S.pombe) Ser-tRNA (SUP12-UCG)...	147	0.0083	1
gb|K03082|YSPTGSS9C	Yeast (S.pombe) intergenic convertan...	147	0.0083	1
gb|K01631|YSPTGSUP	S.pombe intergenic convertant sup3-u...	146	0.0099	1
gb|M10371|YSPTGSM3	Yeast (S.pombe) Ser-tRNA (SUP3-UGA,R...	146	0.010	1
gb|K03079|YSPTGSS3	Yeast (S.pombe) wild-type Ser-tRNA (...	146	0.010	1
gb|K03080|YSPTGSS3C	Yeast (S.pombe) intergenic convertan...	146	0.010	1
gb|J01379|YSPTGSM	Yeast (S.pombe) Ser-tRNA and Met-tRN...	147	0.011	1
emb|X81846|SPHUS5	S.pombe hus5 gene	147	0.012	1

Sequences producing High-scoring Segment Pairs:		Score	P(N)	N
gb|M26293|YSPTGRRA	S.pombe tRNA genes tRNA-Met and tRNA...	146	0.013	1
gb|K01632|YSPTGSGAS	S.pombe UGA suppressor Ser-tRNA gene...	146	0.014	1
emb|X60720|MLTPATT	M.leprae tRNA-Pro gene with homology...	140	0.044	1
gb|L01263|MSGB577COS	M. leprae genomic dna sequence, cosm...	140	0.048	1
gb|M22964|STMSAM2B	S.ambofaciens integrated element pSA...	137	0.051	1
gb|M22966|STMSAM2D	S.ambofaciens integrated element pSA...	137	0.051	1
emb|X16761|MCTRP	Mycoplasma capricolum transfer RNA-P...	128	0.055	1
gb|M27051|SA2ATTBAMB	Plasmid pSAM2 (from S.ambofaciens) a...	137	0.066	1
gb|M27049|SA2ATTB	Plasmid pSAM2 (from S.lividans) DNA ...	137	0.069	1
gb|L21973|MUSE2F1A	Mus musculus E2F1 gene, complete cds.	132	0.21	1
emb|X54496|HSDYS3UA2	H.sapiens 3'region of dystrophin gen...	111	0.22	1
gb|K00314|SOBTRMI	S.obliquus initiator Met-tRNA-i.	121	0.24	1
emb|Y00359|CHZMR14	Maize chloroplast DNA for ribosomal ...	130	0.28	1
emb|X16619|CRARG7	Chlamydomonas reinhardtii ARG7 gene ...	130	0.30	1
emb|Z24107|HS291ZA9	H. sapiens (D14S275) DNA segment con...	128	0.32	1
gb|K00307|HALTRMI	Halobacterium volcanii initiator Met...	120	0.36	1
emb|X03199|HCMET	Halobacterium cutirubrum transfer RN...	120	0.36	1
emb|Z11839|TMNUSGGE	T.maritima nusG gene and genes for r...	128	0.40	1
emb|X55910|GSGSE12G	G. senegalensis GSE12 (SINE family) ...	119	0.70	1
gb|M20946|MPOCPTRNA	Liverwort chloroplast Asn-tRNA-gtt.	114	0.71	1
emb|X01016|CHNT09	Tobacco chloroplast genes for tRNA-P...	123	0.73	1
gb|M26978|MEFTRNAB	Methanothermus fervidus Asn-, Met-, ...	123	0.74	1
gb|M20969|MPOCPTRWA	Liverwort chloroplast Trp-tRNA-cca.	114	0.76	1
emb|X07366|CHZMTPW	Maize plastid DNA for tRNA-Pro and t...	122	0.77	1
emb|X79062|MMHEXA214	M.musculus HEXA gene, exons 2 - 14	107	0.77	2
gb|J04502|MZECPPETE	Maize chloroplast cytochrome b559 al...	122	0.81	1
gb|L14300|HUMMFD293	Homo sapiens (chromosome 1) DNA sequ...	121	0.83	1
gb|T08835|T08835	EST06727 Homo sapiens cDNA clone HIB...	120	0.87	1
emb|X15245|LDTRNA1	Lactobacillus delbrueckii 5S rRNA ge...	120	0.90	1
gb|M26977|MEFTRNAA	Methanothermus fervidus Pro-, Thr-, ...	120	0.90	1
emb|X55914|GSGSE36G	G. senegalensis GSE36 (SINE family) ...	118	0.90	1

Sequences producing High-scoring Segment Pairs:		Score	P(N)	N
gb|M32623|HUMPSBGA02	Human pregnancy-specific beta-1-glyc...	120	0.91	1
emb|X14385|ALCRPEF	Astasia longa chloroplast rps7 and t...	120	0.92	1
emb|X75652|ALRIBT	A.longa plastid genes for tRNAs, rib...	120	0.92	1
emb|X16751|MCTRI1	Mycoplasma capricolum transfer RNA-I...	112	0.93	1
emb|X55106|MLTRNAP1	M. luteus gene for tRNA-Pro (UGG)	118	0.95	1
gb|K00316|NEUTRMI	N.crassa initiator Met-tRNA-i.	111	0.95	1
emb|X03334|GSE36	Galago senegalensis Monomer family S...	118	0.96	1
emb|X05893|DMACHE	Drosophila melanogaster mRNA for ace...	118	0.97	1
emb|X17572|DMACETRNA	D.melanogaster RNA for acetylcholine...	118	0.98	1
emb|Z24254|HS304XF5	H. sapiens (D19S416) DNA segment con...	117	0.98	1
gb|L38981|SHPMSMCMF	Ovis aries microsatellite repeat MCM...	116	0.99	1
gb|M95694|MUSR55SQ	Mouse microsatellite.	115	0.993	1
dbj|D13102|RICMT06	Rice mitochondrial DNA for tRNAs. >e...	116	0.994	1
emb|Z23367|HS113XA9	H. sapiens (D16S501) DNA segment con...	114	0.995	1
emb|X55315|MMNFYA	M.musculus mRNA for CAAT-box DNA bin...	116	0.996	1
emb|A00383|A00383	H.sapiens NANB-hepatitis associated DNA	116	0.996	1
emb|A00970|A00970	NANB-hepatitis associated DNA	116	0.996	1
emb|A00384|A00384	H.sapiens NANB-hepatitis associated ...	116	0.996	1
emb|A00971|A00971	NANB-hepatitis associated DNA, Rever...	116	0.996	1
emb|X53411|HNABV	New Hepatitis non-A, non-B associate...	116	0.996	1
emb|X05603|CHTATRNW	Wheat chloroplast DNA for transfer R...	115	0.996	1
emb|Z16630|HS123XH2	H. sapiens (D12S86) DNA segment cont...	114	0.996	1
gb|U00021|U00021	Mycobacterium leprae cosmid L247.	116	0.996	1
emb|Z31091|MMTEST27	M.musculus expressed sequence tag MT...	114	0.998	1
gb|U15703|OAU15703	Ovis aries OarCP73 gene microsatelli...	113	0.998	1
gb|M94625|PTECPTRA	Porphyra umbilicalis chloroplast tra...	115	0.999	1
gb|M57678|HUMHLL4G	Human hll4 gene, complete cds.	115	0.999	1
emb|X05602|MITATRNW	Wheat mitochondrial DNA for transfer...	114	0.999	1
emb|Z23897|HS267ZD5	H. sapiens (D14S269) DNA segment con...	114	0.999	1
gb|L19543|RATSATEA	Rattus norvegicus (clone D8N228) DNA...	112	0.9996	1
emb|X65949|ECSHITOX	E.coli gene for variant of Shiga-lik...	114	0.9997	1

Sequences producing High-scoring Segment Pairs:		Score	P(N)	N
emb|X13704|ZMTRNWP	Maize linear plasmid mitDNA for tRNA...	114	0.9997	1
emb|Z16662|HS143XH10	H. sapiens (D4S400) DNA segment cont...	113	0.9997	1
gb|K02901|RATIGCA	Rat Ig germline epsilon H-chain gene...	114	0.9997	1
gb|S46131|S46131	dopamine D1 receptor {promoter} [rat...	114	0.9997	1
gb|J05677|RATGCA	Rat guanylyl cyclase A/atrial natriu...	114	0.9997	1
gb|K00358|SPICPTRP	Spinach chloroplast Pro-tRNA.	107	0.9997	1
gb|L26713|MUS164A	Mus musculus expressed sequence tag ...	113	0.9998	1
gb|M98787|HUMD3S124X	Human dinucleotide polymorphism at t...	112	0.9998	1
emb|X66055|HSMS572	H.sapiens polymorphic microsatellite...	112	0.9998	1
emb|Z16587|HS109YA3	H. sapiens (D14S66) DNA segment cont...	112	0.99995	1
gb|F03182|HSC10G082	H. sapiens partial cDNA sequence; cl...	112	0.99995	1

Appendix C :

Table of amino acid symbols

Amino Acid	Symbol
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Appendix D :

Research communications

Some of these results (Chapter 3) have been presented in poster form at the 7th International Symposium on Plant Microbe Interactions, held in Edinburgh in June 1994:

O'Connell, M. and Devine, M. (1994). Ammonium transport in *Rhizobium meliloti*. 7th International Symposium on Plant Microbe Interactions (Abstracts), p28, no.55.

Results detailed here have also been published in the following report:

O'Connell, M. and Devine, M. (1994). Evidence for active ammonium uptake in *Rhizobium meliloti*. *In* Proceedings of EC sectional meeting on Plant-Microbe Interactions, Paris, April 1994.