PHYSIOLOGICAL AND GENETICAL ANALYSIS
OF
THE DEREPRESSION OF NITROGENASE ACTIVITY
IN *PARASPONIA-RHIZOBIUM* STRAIN ANU289

A THESIS SUBMITTED FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

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BY

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Unless otherwise stated, this thesis embodies the independent research work carried out in Botany Department, Faculty of Science and Genetics Department, Research School of Biological Sciences at the Australian National University. Some results reported in Chapter 8 were obtained at the C.F. Kettering Research Laboratory, Yellow Springs, USA.

S.S. Mohapatra
The scientist's dream may be of fields of cereal, unencumbered by symbiotic bacteria, fixing nitrogen with their own nitrogenase, regulated in economical concordance with photosynthesis and lasting from seedling to seed formation. The plants would be tolerant of cold and water stress, with nif stably integrated into their genome, and nif would cause no more drain on the plant economy than exogenous nitrate; there would be fewer environmental problems from run-off, because nif expression would cease at seeding........ and so on!

- JRP
This thesis is dedicated to

TULU (NANA) and DEGAR

Two dearest friends who passed away
for their heavenly abode
during the tenure of this
investigation.
ACKNOWLEDGEMENTS

I take this opportunity in expressing my deep sense of gratitude and sincere regards to Dr Peter M. Gresshoff for his enthusiastic encouragement, advice and criticism throughout this project. Dr Barry Rolfe (Genetics, Research School of Biological Sciences) is sincerely thanked for his interest in the project and help in criticism and discussion during the tenure of this investigation.

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I am greatly indebted to my mother and family whose inspiring words have kept me active.

Finally, the postgraduate research scholarship awarded by the Australian National University is gratefully acknowledged.

Shyam S. Mohapatra
ABSTRACT

Rhizobium strain ANU289, capable of effective nodulation and symbiotic nitrogen fixation in some tropical legumes (such as Macroptilium atropurpureum (siratro)) and the tropical non-legume Parasponia, was found to be derepressable under defined in vitro conditions. This permitted an analysis of the physiological and genetic parameters, which control nitrogenase activity.

Acetylene reduction of strain ANU289 was detected on agar slopes, liquid stationary culture and rapidly agitated liquid culture. The derepression of nitrogenase activity was further optimised using a range of nutritional conditions involving carbon sources, nitrogen sources, molybdenum, iron and cyclic nucleotides. From comparison with other cowpea Rhizobium strains (such as 32H1 and C8756) it was shown that strain ANU289 differed in some requirements for optimal nitrogenase activity.

Employing the mutant analyses three major pathways controlling nitrogenase activity were investigated. These included carbon metabolism (e.g. EPS synthesis and succinate metabolism), nitrogen metabolism (e.g. ammonium and glutamine assimilation) and energy metabolism (e.g. hydrogen uptake and electron transport system). A negative correlation between EPS synthesis and the development of nitrogenase activity in vitro was demonstrated both at the genetic and physiological level. Metabolic systems such as succinate utilization, hydrogen uptake, and electron transport played a positive role in stimulating nitrogenase activity both in vitro and in planta.
Compared to other cowpea rhizobia nitrogenase activity in strain ANU289 was more sensitive to inhibition by exogenous ammonium and glutamine. The mechanism of glutamine inhibition remained obscure. The strain was capable of glutamine uptake and possessed a glutaminase enzyme system. The analysis of mutants defective in glutamine assimilation indicated a negative role of glutamine assimilation in nitrogenase activity.

In common with many other rhizobia oxygen was required for the development of nitrogenase activity. But the activity was sensitive to higher oxygen concentration (more than 10% \( \text{O}_2 \) in the gas phase in stationary liquid cultures and 0.25% \( \text{O}_2 \) in rapidly agitated liquid cultures). In contrast, nitrogenase activity in detached nodules of Parasponia and siratro induced by strain ANU289 showed differential sensitivity to \( p\text{O}_2 \). This led to a study of cytochromes in cultured cells and bacteroids of strain ANU289 and a preliminary analysis of hemoglobin in siratro and Parasponia nodules. The role of these cytochromes and hemoglobin in nitrogen fixation in Parasponia is discussed.

The analysis of these results integrated with in vitro studies of other derepressable Rhizobium strains and the studies with Parasponia-Rhizobium symbiosis have permitted to postulate on the requirements for efficient nitrogenase activity in Rhizobium strains in general and specifically in Parasponia nodules.
PUBLICATIONS


Manuscripts in preparation:


Abstracts accepted:


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<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>Assm</td>
<td>assimilation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BMM</td>
<td>Bergersen's modified medium</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>Cyt</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethylmethane sulfonate</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polysaccharides</td>
</tr>
<tr>
<td>Ets</td>
<td>Electron transport system</td>
</tr>
<tr>
<td>fix</td>
<td>fixation - expression nif genes in plants</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase (EC 1.4.1.4)</td>
</tr>
<tr>
<td>GOGAT</td>
<td>L-glutamine: 2-oxoglutarate aminotransferase (EC 2.6.1.53)</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase (EC 6.3.1.2)</td>
</tr>
<tr>
<td>HEPES</td>
<td>[4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid]</td>
</tr>
<tr>
<td>Hup</td>
<td>hydrogen uptake (uptake hydrogenase EC 1.12.1.2)</td>
</tr>
<tr>
<td>Km</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LBG</td>
<td>Luria broth with glucose</td>
</tr>
<tr>
<td>MES</td>
<td>[2 (N-morpholino)-ethane sulfonic acid]</td>
</tr>
<tr>
<td>MOPS</td>
<td>(3-[N-Morpholino] propanesulfonic acid)</td>
</tr>
</tbody>
</table>
Nitrosoguanidine

Structural genes coding for enzyme nitrogenase (EC 1.7.99.2),
nif⁺ = expression of nif genes in culture

Neomycin

nodulation

Nitrogen regulation

Poly-β-hydroxybutarate

(Piperazine - N, N'-bis-2-ethanesulfonic acid)

Rifampicin

Rhizobium growth medium

Streptomycin

succinate - succinate utilization genes

(N-[tris-(hydroxymethyl)-methyl] glycine)

Trizma base

Trifolii medium with yeast extract

Transposon 5

Triphenyl tetrazolium chloride

volume per volume

weight per volume

weight per weight
CHAPTER-1

GENERAL INTRODUCTION

Research is an organised method for keeping you reasonably dissatisfied with what you have. - C.F. Kettering
1. GENERAL INTRODUCTION

1.1 Biological nitrogen fixation

Symbiotic nitrogen fixation, at present, is not only of immense economic and agricultural importance but also of fascinating academic interest as it involves intimate interactions between prokaryotes and eukaryotes. The central biochemical reaction, which constitutes the conversion of atmospheric nitrogen to ammonia, is catalysed by the enzyme nitrogenase. The enzyme is found only in prokaryotes such as bacteria and cyanobacteria (see review by Stewart 1982).

Of particular interest is the distinction between asymbiotic and symbiotic nitrogen fixation. Asymbiotic nitrogen fixation is widely scattered throughout many prokaryotic genera but can be further classified into four different groups, namely, anaerobic (e.g. Clostridium), facultative (e.g. Klebsiella), microaerobic (e.g. Azospirillum, some Rhizobium strains), and aerobic (e.g. Azotobacter). These free-living microbes not only make a substantial agronomic contribution (Subha Rao 1980), particularly in poorer unfertilized soils, but more importantly have been used in an indepth analysis of the structure, function and regulation of nitrogenase expression.

Symbiotic nitrogen fixation is manifested either as a loose association as seen with members of plant family Gramineae (Dobereiner and Boddey 1981) or as a more complex association of mutual benefit, for instance the root or stem (as in Sesbania, Dreyfus and Dommergues 1981) nodules. These tightly co-ordinated associations occur between a wide range of higher plants including aquatic ferns like Azolla (Peters et al. 1981) with the members of the genera Rhizobium, Frankia, Anabaena or Nostoc. Of these principal nitrogen-fixing systems, symbioses involving Rhizobium as the microsymbiont are of paramount importance in global agriculture.
1.2 Rhizobium-legume symbioses

In terrestrial habitats the legume symbioses account for the largest contribution of fixed nitrogen (Atlas and Bartha 1981). This is due to the size and diversity of the legume order with an estimated 16-19,000 species (Allen and Allen 1981) and the highly efficient nature of the symbiosis (up to 500kg N₂/ha can be fixed by certain legumes). The ability to form nitrogen fixing nodules is greatest within the subfamily Papilionoideae of the Leguminoseae. Based on criteria associated with reproductive structures and specific geographical regions various tribal relationships have been suggested in this sub-family. Thus the largely tropical/sub-tropical Phaseoleae, Desmodieae and Indigofereae are infected by slow-growing rhizobia such as *R. japonicum*, *R. lupini* and *Rhizobium* sp. (also referred to as 'cowpea miscellany', *R. vigna*). In contrast the Viciieae and Trifolieae are mainly temperate and are infected with fast-growing rhizobia such as *R. trifolii*, *R. leguminosarum*, *R. meliloti* and *R. phaseoli*. A comparison in respect of symbiotic characteristics between these tropical, sub-tropical versus temperate tribes infected with their corresponding *Rhizobium* species is presented in Table 1.1.

Nitrogen fixation in legume nodules occurs as a function of *Rhizobium* enzyme 'nitrogenase'. The role of the plant is to furnish the necessary nutritional and physiological environment (Gresshoff et al. 1981). Development of nitrogen-fixing nodules comprises a multistep cascade of events, namely, rhizobial root colonisation, root hair adhesion, hair curling and branching, infection thread formation, nodule initiation and development, bacteroid development and nitrogen fixation (Vincent 1980). This requires a tightly co-ordinated read-off of genetic signals between plant and *Rhizobium* (see review by Verma and Long 1983). The isolation of different classes of bacterial mutants unable to carry
Table 1.1. Comparison between tropical/sub-tropical versus temperate legume tribes in respect of nodule, bacteroid and free-living Rhizobium characteristics

<table>
<thead>
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<th>Vicieae/Trifolieae</th>
<th>Desmodieae/Phaseoleae</th>
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<tbody>
<tr>
<td><strong>Nodules:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nature</td>
<td>indeterminate</td>
<td>determinate</td>
</tr>
<tr>
<td>Vascular transfer cells</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td>Infected cells</td>
<td>vacuolated</td>
<td>non-vacuolated</td>
</tr>
<tr>
<td>Principal export</td>
<td>soluble amides</td>
<td>insoluble ureides</td>
</tr>
<tr>
<td></td>
<td>asparagine/glutamine</td>
<td>allantonic acid</td>
</tr>
<tr>
<td>Vascular system</td>
<td>open ended, branched</td>
<td>closed, branched loop from the stele of the subtending root.</td>
</tr>
<tr>
<td><strong>Bacteroids:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA content</td>
<td>higher than free-living form</td>
<td>similar</td>
</tr>
<tr>
<td>Cell wall</td>
<td>major cell wall change</td>
<td>no change</td>
</tr>
<tr>
<td>Pleiomorphism</td>
<td>enlarge greatly and become markedly pleiomorphic</td>
<td>enlarge slightly and swell, not markedly pleiomorphic</td>
</tr>
<tr>
<td>Detergent treatment</td>
<td>sensitive</td>
<td>resistant</td>
</tr>
<tr>
<td>Viability</td>
<td>little</td>
<td>some</td>
</tr>
<tr>
<td><strong>Free-living rhizobia:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth rate on agar</td>
<td>fast</td>
<td>slow</td>
</tr>
<tr>
<td>Tolerance of acid</td>
<td>poor</td>
<td>good</td>
</tr>
<tr>
<td>Nitrogenase in vitro</td>
<td>not substantiated</td>
<td>recorded in many strains</td>
</tr>
<tr>
<td>Ease of genetic study</td>
<td>easy, frequent</td>
<td>difficult and rare</td>
</tr>
<tr>
<td>Host range</td>
<td>restricted (?) coupled with tendency towards self fertilization (8-10°C)</td>
<td>wide coupled with tendency for cross pollination (16-30°C)</td>
</tr>
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out the symbiotic process has demonstrated that a number of bacterial
genes are involved in the establishment of nitrogen-fixing nodulation
(Rolfe et al. 1981). Using transposon mutagenesis, it has been possible
to identify a spectrum of mutant root-nodule phenotypes, ranging from no
nodule at all (Nod⁻) to nodules that appear morphologically normal but
fail to fix nitrogen (Fix⁻). Spectacular advance has been made in
analysis of these genes particularly in fast-growing rhizobia such as R.
trifolii (Hughes 1982, Scott et al. 1982), R. meliloti (Long et al. 1982,
Weber and Pühler 1982), and R. leguminosarum (Johnston et al.
1983) and also in the slow-growing R. japonicum (Hennecke 1981).

In contrast, knowledge of the genetic contribution of the
counterpart to symbiotic nitrogen fixation is very limited. Through
conventional genetic experiments, several genes in clover, soybean and
pea have been found to be involved in nodulation (see review by Caldwell
and Vest 1977, Holl 1983). In addition, there is now significant genetic
as well as physical evidence (Legocki and Verma 1980, Verma et al. 1981)
from studies in soybean - R. japonicum interactions, that a number of
host genes are involved in nodule development. More recently, Van den Bos
et al. (1983) have reported about 25 different nodulins (nodule specific
proteins) which appear differentially at various phases of nodule
development in pea. To date, two nodule specific proteins, leghaemoglobin
(Lb) and nodulin-35 (uricase) have been well characterised.

1.2.1 *Rhizobium* - nonlegume symbioses

Non-legume nodulation is widespread among several families of
angiosperms. Most of these involve the actinomycete Frankia as
microsymbiont (Quispel and Burggraaf 1981). Very few reports on non-legume
symbioses claim *Rhizobium* involvement, as it was previously thought that
*Rhizobium* only nodulates legumes (Buchanan and Gibbons 1974). Several
studies, of which a few are described here, have examined Rhizobium induced nodulation in non-legumes. Although one of these has been confirmed, all others have not yet been through rigorous testings required for confirmation. For example, Koch's principles should be satisfied and modern methods of measurement of nitrogen fixation such as acetylene reduction or $^{15}\text{N}$ incorporation should be used.

Certain Egyptian xerophytic shrub species such as Zygophyllum album, Z. decumbens, Z. simplex, Z. coccineum, Fagonia arabia and Tribulus alatus were reported to possess nitrogen-fixing root nodules containing rhizobia-like cells. The bacterial isolate was shown to nodulate peanuts (Sabet 1946, Mostafa and Mahmoud 1951); however, there has been some criticism of such studies (Allen and Allen 1958). Recently, 5 related species of Zygophyllum, namely T. terrestris, T. longipetalous, Z. simplex, Z. pripinquium and F. indica found in Pakistan were reported to bear root nodules (Athar and Mamood 1981). A detailed examination of T. terrestris shows that these nodules structurally resemble actinorhizal-type nodules, whereas the endophyte appears more like R. japonicum. The isolated bacterium is slow-growing and is able to nodulate and fix nitrogen in several legumes. However, as stated before, none of these results have either been conclusive or led to a continuum of scientific research and thus could be referred to as 'phenomenal'.

Of significance in the present context and one of the most interesting and exciting discoveries of recent years is the non-legume genus 'Parasponia', which establishes a nitrogen-fixing symbiosis with Rhizobium. This symbiotic system is described in more detail in the following section.
1.2.2 *Rhizobium - Parasponia* symbioses

Root nodules were first observed on *Parasponia*, a tropical pioneer plant family found in parts of the Malay Archipelago, by Ham (1909). But it was Trinick (1973) who showed that the microsymbiont was *Rhizobium*. Trinick (1973), identified the host as *Trema aspera* which was later amended to *T. cannabina* (Trinick and Galbraith 1976) and then finally to *Parasponia rugosa* (Akkermans et al. 1978). Though *Parasponia* and *Trema* are extremely similar in appearance and share the same habitat, to date there are no confirmed reports of nodulation with *Trema* (Akkermans et al. 1978). *Rhizobium* induced nodules have been observed in three other species of genus *Parasponia* namely *P. andersonii* (Trinick 1980), *P. parviflora* (Becking 1979), and *P. rigida* (Tjepkema 1981).

The bacteria isolated from the nodules of these species are morphologically, nutritionally, culturally and serologically similar to slow-growing rhizobia, and are supposedly best classified as part of the 'cowpea miscellany' (Trinick and Galbraith 1980). Isolates from *Parasponia* failed to nodulate most legumes that are usually nodulated by slow-growing rhizobia. Generally in cases where nodulation occurred, the symbiosis was at best partially effective. Of 15 isolates tested, only isolate CP283 (the parent strain of ANU289) was promiscuous and highly effective on a range of tropical legumes namely *Flemingia congesta*, *Glycine wightii*, *Lablab purpureus*, *Macroptilium atropurpureum*, *M. lathyroides*, *Stylosanthes humilis*, *Vigna luteola*, *V. marina* and *V. unguiculata* (Trinick and Galbraith 1980).

*Parasponia* nodules resemble a modified lateral root with a central vascular bundle surrounded by the infected zone. The presence of the apical meristem in the *Parasponia* nodule which differentiates into vascular and cortex tissue producing nodule elongation, is also a feature of legume coralloid nodules. The other major difference between the
legume and Parasponia symbioses lies in the fact that in the legume, the bacteria 'bud off' from the infection thread whilst in Parasponia, the bacteria remain confined to the infection thread, which continues to ramify in host cells and eventually becomes what may be called a 'fixation thread'. However, the confinement of cells to the infection thread is not universal, as species differences exist in regard to bacterial release within the Parasponia genus, partial release being observed in P. parviflora (Becking 1979) and P. rugosa (Trinick and Galbraith 1976). No bacterial release occurs in P. rigida which was used in this study (Price et al. under preparation). Rhizobia in the fixation threads were reported to partially differentiate into bacteroids (Becking 1979) and this is in part substantiated by data of this study (see Chapter 8). Table 1.2 presents a comparison of legume and non-legume nodules produced by Rhizobium infection with that of actinomycetes induced nodules in other non-legumes. The nodule ultrastructure particularly of P. rigida and siratro (Macroptilium atropurpureum) will be partly discussed in Chapter 7 and 9.

Whether or not the discovery of this association between Rhizobium and a non-legume will help the establishment of new symbioses by genetic engineering still remains uncertain. Undoubtedly the 'promiscuity' of this Rhizobium strain and the non-legume genus Parasponia will facilitate the study of many physiological, biochemical and genetical characteristics underlying successful symbiotic nitrogen fixation.

1.3 The nitrogenase system

Nitrogenase is one of the most studied prokaryotic enzymes as evidenced by vast scientific literature (see reviews Brill 1980, Roberts and Brill 1981, Stewart 1982). The central reaction catalysed by
Table 1.2. Comparison of some symbiotic characters among legume-Rhizobium, Parasponia-Rhizobium and non-legume-actinomycetes symbioses

<table>
<thead>
<tr>
<th>characters</th>
<th>Legume-Rhizobium</th>
<th>Parasponia-Rhizobium</th>
<th>Non-legume-actinomycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsymbionts</td>
<td>gram-negative bacteria</td>
<td>gram-negative bacteria</td>
<td>gram-positive actinomycetes</td>
</tr>
<tr>
<td>Nodule phenotype</td>
<td>globose or cigar shaped</td>
<td>coraloid</td>
<td>coraloid</td>
</tr>
<tr>
<td>Vascular bundle</td>
<td>lateral, surround outer extremities of the infected zone</td>
<td>central, resemble a modified lateral root</td>
<td>central, also resemble a modified lateral root</td>
</tr>
<tr>
<td>Inner cortical cell layer void of air space</td>
<td>present</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td>Infected zone</td>
<td>plant cells filled with bacteroid(s) in peribacteroid membrane envelopes</td>
<td>usually plant cells filled with infection threads where bacteroids are present in a membrane envelope</td>
<td>infection threads contain the vesicles</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>monomeric leghemoglobin cross react with myoglobin</td>
<td>dimeric hemoglobin with no cross reaction</td>
<td>present but its nature unsubstantiated</td>
</tr>
<tr>
<td>Energy usage (mole of CO₂ evolved per mole of acetylene reduction)</td>
<td>varies from 2.5 to 5.6</td>
<td>3.4</td>
<td>not known</td>
</tr>
</tbody>
</table>
nitrogenase is the same in all organisms so far studied. It is as follows:

\[ \text{Mg}^{++} \]

\[ N_2 + 6e^- + 6H^+ + 12ATP \rightarrow 2NH_3 + 12ADP + 12Pi \]

**Nitrogenase**

To date, nitrogenase has been extracted from all known nitrogen-fixing organisms and has been purified from 6 microbes, including Rhizobium bacteroids (Postgate 1978). Nitrogenase expression in most Rhizobium strains is restricted to the bacteroid state of Rhizobium (but see 1.3.3).

On purification, nitrogenase separates into two distinct proteins, component I and component II, both dark brown in colour, neither active without the other. So far it has not been possible to purify the entire nitrogenase complex as one unit. The larger protein (component I or Mo-Fe Protein, MW 220,000) consists of four subunits, two copies each of the \( \alpha \) and \( \beta \) subunits (coded by \text{nifD} and \text{nifK} genes) each requiring the other for stability in vivo. Though less clearly defined, the Mo-Fe protein has about 4 Fe\( _4S_4 \) clusters (P clusters) and two MoFe co-factors (M centres). It is believed that nitrogen gas (\( N_2 \)) binds to the Mo of Mo-Fe co-factor.

The smaller iron protein (component II, MW 60,000) is composed of two copies of a single subunit coded by \text{nifH} gene and contains a single Fe\( _4S_4 \) cluster. It perhaps plays a role in the supply of electrons. The similarity of the respective component proteins between different microbes and the readiness with which they are destroyed in the presence of oxygen, is suggestive of strongly evolutionary conservation for the molecular properties required for this reaction. For detailed information on these protein components, see the recent reviews Brill (1980) and Stewart (1982).
1.3.1 Requirements for the nitrogenase reaction \textit{in vitro}

For nitrogenase to function there is an absolute requirement for ATP, for a reductant supply, and for Mg$^{2+}$. This combines with ATP to produce a MgATP complex which may bind to the Fe protein in such a way that electron transfer from this protein to MoFe protein occurs (Burris et al. 1980). In addition to N$_2$, nitrogenase can reduce a wide variety of substrates and its ability to reduce acetylene has greatly facilitated the study and quantification of nitrogen fixation (Dilworth 1966). As well as being involved in reducing a particular substrate, nitrogenase always reduces protons, so that H$_2$ production is a normal concomitant function of the nitrogenase reaction. Proton reduction probably precedes N$_2$ reduction (Thorneley and Lowe 1981).

In cell free preparations, sodium dithionite (Na$_2$S$_2$O$_4$), a non-physiological reducing agent couples directly to nitrogenase; thus an electron carrier (as is needed \textit{in vivo}) is not necessary. Two ATP molecules are hydrolysed for each electron transferred; thus a total of 12 ATPs are used for each molecule of N$_2$ to be reduced to NH$_3$. Therefore, nitrogen fixation is considered to be 'energy expensive'. Since ADP is an inhibitor of nitrogenase activity, an ATP generating system is used in cell-free studies. An ATP/ADP ratio of 10:1 was suggested for maximum nitrogenase activity (Erecinska et al. 1977). Such a cell-free \textit{in vitro} system is outside the scope of this study and will not be discussed further.

Expression of nitrogenase activity in whole cells is more complex in contrast to studies in cell-free preparations for two reasons. Firstly, whole cell activity takes into account not only synthesis and degradation of the enzyme (governed by genetic factors) but also modulation of activity (see section 1.3.2 for a detailed discussion). Secondly, the physiological requirements for nitrogenase functioning are
dependent on general cellular metabolism. As nitrogen-fixing micro-
organisms differ in their physiology, strategies for derepression of
nitrogenase activity vary accordingly (Kennedy 1980). How nitrogenase
fits into the general metabolism in aerobic bacteria is illustrated in
Fig. 1.1. In such a system, carbohydrate-respiration is the usual source
of both electrons and ATP. Electrons generated by glycolysis and TCA
cycle are passed onto nitrogenase via specific electron carrier proteins
such as ferredoxins or flavodoxins. Part of the electrons are made
available for oxidative phosphorylation, a process which produces ATP
required to drive nitrogenase reaction. While some oxygen is necessary
for ATP synthesis, the intracellular free oxygen must be low enough, so
that nitrogenases are not inactivated. To this end, micro-organisms have
evolved various ways to protect nitrogenases from oxygen (see Chapter 7
and 8), while being able to use some oxygen to derive other requirements
(such as ATP) for nitrogenase expression.

1.3.2 Regulation of nitrogenase activity in non-symbiotic free-living
bacteria

Asymbiotic bacteria can be grouped into two main categories based
on the studies on nitrogenase regulation. The first category contains
free-living bacteria such as Clostridium, Klebsiella, Azotobacter and
others. Our present knowledge of the regulation of nitrogenase expression
can be largely attributed to studies with these micro-organisms. These
organisms use fixed nitrogen for their cellular growth.

The other category includes those bacteria, which normally act as
microsymbionts in root nodule symbiosis (e.g. Rhizobium strains) or
associative symbiosis (e.g. Azospirillum) but of which some strains can
derepress nitrogenase activity under microaerobic conditions. Studies
with these latter systems are limited and will be discussed in section
1.3.3.
Nitrogenase is regulated primarily at three levels (Eady 1981, Hochman 1982) which can be temporally separated. One of these with a relatively slow response is the control of intracellular enzyme concentration. The final concentration is a function of derepression of nitrogenase synthesis (mainly through transcriptional control, mRNA half-life) and degradation of pre-existing enzyme. In all free-living diazotrophs, with the exception of Rhizobium, nitrogenase synthesis is repressed by ammonia or its precursors such as nitrate, nitrite and amino acids if present in excess of the bacterial nitrogen requirements (see review by Eady 1981). Repression by oxygen, cyclic nucleotides and carbamoylphosphate is also reported (Mortenson 1978, Brill 1980).

At the second level of regulation, which shows a much faster response, the potential nitrogenase activity in the derepressed state is modulated by various metabolites. Factors which have been implicated in the rapid modulation of activity in vitro (see Eady 1981 for review) include: concentration of ammonium (Mortenson 1978), supply of ATP and the ATP/ADP ratio (Haaker et al. 1974), supply of reducing equivalents the degree of membrane energization (Laane et al. 1980), and the maintenance of sufficiently low dissolved oxygen concentration (Hochman and Burris 1981).

An additional mode of regulation was discovered in the purple non-sulfur bacterium Rhodospirillum rubrum (Ludden and Burris 1976), the photosynthetic bacterium Rhodopseudomonas capsulata (Yoch 1979) and Rhodopseudomonas palustris (Zumft and Castillo 1978) and in the aerobe Azospirillum lipoferum (Ludden et al. 1978). In these organisms, nitrogenase activity was modulated by covalent modification of the Fe-protein. The inactive component can be fully activated by a membrane-bound activating factor. In Rhodopseudomonas, nitrogenase can exist in two interconvertible forms: the 'A' form which predominates in
FIG. 1.1 A SCHEME FOR INTEGRATION OF NITROGENASE IN CELLULAR METABOLISM HETEROTROPIC AEROBES. REACTIONS OF GLYCOLYSIS (GLUCOSE TO PYRUVATE) ARE CATALYZED BY THE ENZYMES OF EITHER EMBDEN-MYERHOF-PARNS (EMP) PATHWAY, ENTNER-DOUDEROFF (ED) PATHWAY OR HEXOSE MONOPHOSPHATE (HMP) PATHWAY, YIELDING 2 ATP/GLUCOSE. FURTHER OXIDATION OF PYRUVATE VIA ACETYL-CoA OCCURS IN THE TRICARBOXYLIC ACID (TCA) CYCLE. THIS CYCLE PERFORMS AN IMPORTANT BIOSYNTHETIC ROLE IN PROVIDING α-KETOGLUTARATE AND OXALOACETATE FOR AMINOACID FORMATION, AND CREATES A DEMAND FOR CARBOXYLATION REACTIONS WITH EITHER PYRUVATE OR PHOSPHOENOLPYRUVATE TO REPLENISH C4 DICARBOXYLIC ACIDS. REPRESENTATIVE COMPONENTS OF RESPIRATORY CHAIN YIELDING ATP ARE ALSO SHOWN. NITROGENASE REACTION IS HEAVILY DEPENDENT ON ELECTRONS AND ATP PRODUCED AS A RESULT OF THESE REACTIONS. AT LEAST 6 ELECTRONS AND 12 ATPS (2 ATPs PER EACH ELECTRON TRANSFERRED) ARE NECESSARY FOR REDUCTION OF ONE MOLECULE OF N2 TO AMMONIA (SEE KENNEDY 1980).
Nitrogen depleted cultures and is converted to 'R', the inactive form, in the presence of glutamate or ammonia. The 'R' form requires the activating factor (see Hochman 1982; also the review by Eady 1981). Nitrogenase A and R are in equilibrium and the A/R ratio is dependent on the nutritional state of the cells.

1.3.3 Nitrogenase expression of *Rhizobium* in the asymbiotic (free-living) state

Prior to 1975, a basic tenet of symbiotic nitrogen fixation between *Rhizobium* and members of the Leguminoseae had been that the expression of nitrogenase activity in the bacteria was restricted to the symbiotic state. It was even proposed that a host factor(s) was essential for completion of the nitrogenase enzyme system in the rhizobia (Dilworth and Parker 1969). Evidence for the diffusability of host factors inducing nitrogenase synthesis was obtained in in vitro association of rhizobia with soybean plant cell cultures (Holstein et al. 1971, Phillips 1974, Child and LaRue 1974). However, all these hypotheses were disproved, first by circumstantial evidence (Dunican and Tierney 1974, Bishop et al. 1975) and then by direct evidence (Pagan et al. 1975, McComb et al. 1975, Kurz and LaRue 1975, Tjepkema and Evans 1975, Keister 1975), when it was possible to derepress nitrogenase in asymbiotic cultures of *Rhizobium*. These discoveries not only showed that the nitrogenase genes are bacterial but opened up new avenues for physiological and genetic analysis of nitrogen fixation in *Rhizobium* (detailed in Chapter 3, 4 and 6).

Because no 'plant signal' was necessary to derepress nitrogenase, it was reasonable to assume given appropriate nutritional and oxygen conditions to any *Rhizobium* strain, it would be possible to derepress nitrogenase in vitro. However, it must be stressed that as yet there are
no reliable method(s) available to derepress nitrogenase activity in all slow-growing strains. Although there are a few isolated reports in the literature on nitrogenase activity with fast-growing rhizobia (Kurz and LaRue 1975, Lorkiewicz et al. 1978, Bedmar and Olivares 1979, Skotnicki et al. 1979), these reports have not been substantiated and requests for cultures of strains used have not received a reply. On the other hand, some fast growing rhizobia belonging to Bradyrhizobium, for example Sesbania-Rhizobium strain, can be derepressed for nitrogenase activity.

Why some Rhizobium strains do derepress, while others do not is not known. Since the strains differ with regard to the requirements for expression of nitrogenase activity (Pankhurst 1981, Agarwal and Keister 1982), it may be that the optimal conditions necessary for derepression of nitrogenase in these strains have not been found. Alternately, derepression of nitrogenase in laboratory cultures may be under genetic control. The results of some relatively recent genetic exchange experiments (Johnston and Beringer 1977) and DNA:DNA hybridization studies (Hollis et al. 1981) indicated that strains labeled 'cowpea rhizobia' and R. japonicum may represent at least two and three different sub-species, respectively. Since many strains belonging to one sub-species of R. japonicum were nitrogenase positive in vitro (Agarwal and Keister 1982), it was thought that nitrogenase derepression in culture may be confined to one sub-species.

R. japonicum strains 61A76 and 110, both capable of forming nitrogen-fixing nodules on soybeans, showed very low (24% total DNA) sequence homology with each other and the organisation of nifKDH genes appeared to be different. Whereas strain 61A24 which formed ineffective nodules on soybeans, showed relatively high (50%) sequence homology to strain 110 and the organisation of nitrogen fixation genes in
these strains were apparently similar (Haugland and Verma 1981). Thus it appears that the genetic basis for in vitro nitrogenase expression (similar to in planta effectivity) may be strain specific. The precise molecular architecture for such differences between strains remains to be worked out.

1.4 Genetic analysis of Rhizobium nitrogenase genes

The last decade has seen considerable progress towards an understanding of the more subtle aspects of the genetics of nitrogenase regulation. Phenomenal progress has been made in the study of the nif genes particularly in K. pneumoniae, which has proved the most suitable organism for such studies (reviewed by Brill 1980).

1.4.1 Location of nif genes

Rhizobium fixes N₂ symbiotically and a mutant with a defect in nitrogenase is unable to fix N₂ either in symbiotic or asymbiotic state. This is clear evidence that the bacterial partner contains the full complement of nif genes (Maier and Bill 1976). Because the amino acid sequence of nitrogenase genes are very highly conserved in evolution, it is possible to identify and clone these genes in Rhizobium using previously sequenced nif genes as hybridization probes. Direct evidence that Rhizobium nif genes are plasmid borne has been obtained by hybridization of cloned Klebsiella nitrogenase genes (nifHDK) to purified plasmid DNA (Nuti et al. 1979, Ruvkun et al. 1980, Prakash et al. 1981). In several species, including R. leguminosarum, and R. trifolii, the plasmids (> 200kb) carrying the nif cluster can be preparatively isolated. Using Klebsiella probes, nif structural genes from R. meliloti (Ruvkun and Ausubel 1980) and R. japonicum (Hennecke 1981) have also been cloned. In R. meliloti, nifHDK are arranged in the same transcriptional order as in K. pneumoniae (Weber and Pühler 1982, Ruvkun et al. 1982).
While **nifH** and **nifD** are closely linked in fast-growing species (Scott et al. 1982), in slow-growing *R. japonicum* (Hennecke 1983) and in Rhizobium sp. strain ANU289 (Scott et al. 1983), these are encoded in separate operons, suggesting non-co-ordinate control of Fe and MoFe proteins (see also Shaw 1983). Little is known regarding the regulation of these genes as no other Rhizobium control gene has yet been identified.

### 1.4.2 Transposon (Tn5) mutagenesis and clustering of **nif** genes

The original transposon mutagenesis procedure (also called 'suicide plasmid transfer', Beringer et al. 1978) involves the transfer from *Escherichia coli* into Rhizobium (recipient) of a wide host range P-type plasmid carrying Tn5 (a 5.7 kb transposon that confers Kanamycin (Km) or Neomycin (Nm) resistance). The suicide plasmid also contains an integrated bacteriophage *mu* that causes plasmid instability in Rhizobium host cells. By selecting for KmR/NmR transconjugants, colonies can be selected in which Tn5 has survived the original vector plasmid suicide by transposing into the host cell genome, causing insertional mutation(s). The transposon itself thus acts as a 'tag' for the genetic and physical location of the affected gene. A fine structure genetic analysis is possible by using a site directed-, saturation- or replacement- Tn5 mutagenesis technique (Ruvkun et al. 1981, Ausubel 1982, Corbin et al. 1982). Tn5 mutagenesis using different strategies and plasmids is now widely used in isolation of mutants defective in **nod**, **nif** or heme biosynthesis (Leong et al. 1982) as well as in carbon and nitrogen assimilation as with the present study.

Adopting a strategy designed to replace wild-type **nif** genes in the *R. meliloti* genome with homologous cloned sequences carrying Tn5 insertions, Ruvkun et al. (1982) have analysed the region surrounding the **nifHDK** genes. In a total of 31 *R. meliloti* strains, this Tn5 containing
14kb region showed at least two clusters of symbiotic genes (approx. 6.3kb and 5.0kb). Furthermore, this 6.3kb cluster is a single transcriptional unit that contains the nifH gene at the 5'end. In another R. meliloti strain, studies using saturation mutagenesis have indicated that there are at least three separate units of gene expression spread over a region of 11.2kb. Thus the nif regulatory genes may be clustered in R. meliloti as in K. pneumoniae.

1.4.3 Regulation of nitrogenase expression by other genes

In bacteroids of R. leguminosarum (Van den Bos, pers. comm.) nitrogenase synthesis is not repressed by high O_2 (100µM) or ammonium concentration (20mM). This suggests that in these bacteroids, the so-called 'redox control' and 'nitrogen control' seen in Klebsiella is not operational and the nitrogenase synthesis is constitutive. However, there exists some indirect evidence that nitrogenase expression in Rhizobium may be regulated by other genes. For instance, glutamine synthetase defective mutants (gln⁻) of Rhizobium are nif⁻ both ex planta (in strain 32H1 only) and in planta (Ludwig and Signer 1977, Kondorosi et al. 1977). Two plasmid located genetic functions namely exopolysaccharide (eps, Chakravorty et al. 1982) and uptake hydrogenase (hup, Lepo et al. 1981) have been implicated in affecting nitrogen fixation as have been succinate non-utilizing mutants (Ronson et al. 1981, Glenn and Brewin 1981). Whether these genes exert their effects on nitrogenase synthesis and/or activity or they merely affect availability of other factors essential for maintenance of nitrogenase activity is not known.
5.1 Aims of this study

The aim of this study was to utilize genetic conditionality on the bacterial side to investigate the physiological factors controlling expression of nitrogenase activity in *Rhizobium* strain ANU289. This strain is capable of nitrogen fixation in a wide array of physiological environments namely in *in vitro* conditions, in a range of tropical legume species and the non-legume *Parasponia*.

The primary goal behind the study of these factors regulating nitrogenase activity was to understand what makes this strain ANU289 so efficient in *Parasponia*. Why, for instance, was fixation possible in the infection (fixation) thread, whereas in a legume such a phenotype (defective in bacterial release, bar~) was fix~ (Hughes 1982)? How could *Parasponia* fix nitrogen, when Coventry et al. (1976) found no leghemoglobin in its nodules? What regulates nitrogenase expression in such an organism? Two following lines of study were undertaken to answer some of these questions.

A molecular approach dealing with cloning and sequencing of nif structural genes and other nif regulatory studies were initiated (Scott 1983, Weinman pers. comm.), while this study aimed to delineate physiological and genetical parameters controlling nitrogenase expression in *in vitro* conditions.

The following chapters of this thesis are arranged such that:

Chapter 2 contains the general materials and methods used in this study.

Chapter 3 deals with preliminary attempts to demonstrate *in vitro* nitrogenase activity in a few *Parasponia* strains. The basic requirements for derepression while using different techniques were investigated.
Chapter 4 describes further optimisation of in vitro nitrogenase activity by altering nutritional conditions with a range of sugars, organic acids, nitrogen sources. It also investigates the effects of iron, molybdenum, and cyclic nucleotides.

Chapter 5 looks at regulation of nitrogenase expression using mutants in three different aspects of carbon and energy metabolism. These included exopolysaccharide synthesis, succinate metabolism and uptake hydrogenase systems.

Chapter 6 contains the experiments on control of nitrogenase expression by several nitrogenous compounds and particularly ammonium and glutamine. The study of the relationship between glutamine assimilation and nitrogenase activity employing mutants defective in glutamine utilization was initiated.

Chapter 7 examines oxygen requirements and sensitivity of nitrogenase activity both in in vitro and in detached nodules of siratro and Parasponia.

Chapter 8 extends further the findings of Chapter 7 to study of cytochromes and oxidases in cultured cells as well as bacteroids from siratro and Parasponia nodules. Employing mutants defective in electron transport systems, the role of cytochromes/oxidases in nitrogen fixation was examined. The study is concluded by a preliminary comparative study of hemoglobins in both host types.

Chapter 9 integrates the findings of this study and focuses on two aspects, one being the parameters controlling in in vitro expression of nitrogenase activity in strain ANU289 and the other expounding the Rhizobium Parasponia association as a model symbiotic system. Scope of this study and the directions for future research are included.
CHAPTER-2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains

The bacterial strains used are listed in Table 2.1, with their genetic markers.

Three different Escherichia coli strains were used as transconjugants for transfer to the donor in transconjugant mating. They are as follows:

1. E. coli strain BL21 carrying the pET32a plasmid containing the bla gene, obtained originally from Dr. J. Bergman (U. W. I.)

2. E. coli strain DH5α carrying the pUC18 plasmid (pBR322 backbone) containing the apr gene, obtained originally from Dr. J. Bergman, University of Arkansas.

3. E. coli strain AG1117 carrying the pUC18 plasmid (pBR322 backbone) containing the apr gene, obtained originally from Dr. J. Bergman, University of Arkansas.

2.1.3 Plants

The tropical lagoon strain Chlamydomonas reinhardtii was used for lagoon inoculation tests. Seeds were obtained from Arthur Hargreaves, Pty Ltd., Redfern, New South Wales. Nostoc strains mentioned in section 2.1.1 were also capable of effective inoculation with lagoon populations. The seeds of which were kindly supplied by Dr. K. J. Kelly, the Cunningham Laboratory, Division of Tropical Crops and Pastures, Queensland.

Fernanopsis rufida seeds were provided by Paul J. D. Cowling, Harvard Forest, Harvard University, Petersham, Massachusetts.
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Rhizobium strains used in these studies are listed in Table 2.1 with their genetic markers.

Three different *Escherichia coli* strains were used as transposon Tn5 donors in transposon mutagenesis. They are as follows:

1. *E. coli* strain 1830, carries plasmid pJB4JI which contained the Tn5, obtained originally from Dr. J. Beringer (U.K.)

2. S380 (JMP575) = *E. coli* c600, pSP601 (R751 derivative containing transposon Tn5 (KmR), Tn1ApR), Tn402 (TmR), Tn1771 (TcR), phage muC+ obtained from Prof Dr. A. Pühler, University of Bielefeld.

3. ANU1041 = *E. coli* c600, recA-, (thi, thr, leu, tonA, lac y, supE) contain pSUP201 (pBR325 Mob) RP4.2.Tc:: Mu integrated in the chromosome, obtained originally from Dr. R. Simons, University of Bielefeld.

2.1.2 Plants

The tropical legume siratro (*Macroptilium atropurpureum*) was used for legume nodulation tests. Seeds were obtained from Arthur Yates Co. Pty Ltd, Melpessa, New South Wales. Rhizobium strains mentioned in section 2.1.1 were also capable of effective nodulation in *Lablab purpureus*, the seeds of which were kindly supplied by Dr. R.A. Date, the Cunningham Laboratory, Division of tropical crops and pastures, Queensland.

*Parasponia rigida* seeds were provided by Prof. J. D. Tjepkema, Harvard Forest, Harvard University, Petersham, Massachusetts.
Table 2.1 Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Markers</th>
<th>Original Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU289</td>
<td>CP283 muc⁻, Sm⁺, Rif⁺, nod⁺, fix⁺</td>
<td>Dr M. J. Trinick Division of Land and Resource Management, CSIRO, Perth.</td>
</tr>
<tr>
<td>ANU288</td>
<td>CP283, muc⁺, Sm⁺, Rif⁺, nod⁺, fix⁺</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>NGR231</td>
<td>Sm⁺, nod⁺, fix⁺</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>CB756</td>
<td>Sp⁺, nod⁺, fix⁺, fix⁻ p</td>
<td>Dr A.H. Gibson Division of Plant Industry CSIRO, Canberra.</td>
</tr>
<tr>
<td>32H1</td>
<td>Sp⁺, nod⁺, fix⁺, fix⁻</td>
<td>&quot; &quot;</td>
</tr>
</tbody>
</table>

All strains listed in Table 2.1 belong to the 'Cowpea Miscellany' group.

Abbreviations:

- Sm = streptomycin
- Rif = rifampicin
- Sp = spectinomycin
- nod = nodulation
- fix = nitrogen fixation
- R = resistant
- S = siratro
- P = Parasponia
- muc = mucoid (produces smooth concentric colonies on mannitol containing plates)
- + = ability
- - = inability
2.1.3 Gases

High purity grade gases from CIG (Australia) were always used to ensure that levels of contaminating \( \text{O}_2 \), \( \text{CO} \), \( \text{CO}_2 \) were kept to a minimum.

2.1.4 Other chemicals

All chemicals used for storage, growth, derepression and mutagenesis media in both bacteriological and plant culture studies were of reagent grade. Where commercial preparations have been used, the product and the manufacturer are sited. \(^{14}\text{C}-\text{glutamine} \) was obtained from Radiochemical Centre, Amersham, U.K.

2.2 Media Preparation

For all solid media, 1.5% Difco Bacto-agar was used. All media components (except for heat-labile components) were sterilized by autoclaving together at 15 psi for 15–30 min depending on volume. For example, L-glutamine was always freshly prepared and filter sterilised. All carbon source solutions were filter sterilized before being added to the autoclaved media which was cooled down to 45–50°C. For plates, the media were poured into 99mm diameter sterile, disposable plastic petri dishes holding 25–30ml (Disposable Products, Australia). All post-sterilization manipulations were made in a laminar flow-hood (Standardised Protection, Australia). The preparation of most commonly used stock solutions and media, their compositions and final concentrations are described.
2.2.1 Common stock solutions

Iron chelate: (Gresshoff and Doy 1974)

200 x stock

FeSO\textsubscript{4} 7H\textsubscript{2}O : 557 mg
Na\textsubscript{2} EDTA : 745 mg
Water : 100 ml

heated to dissolve if necessary and stored at 0-4°C.

Vitamins: (Gresshoff and Doy 1974) 100x stock

meso-inositol : 1000 mg
thiamine HCl : 100 mg
nicotinic acid : 10 mg
pyridoxine HCl : 10 mg
Water : 100 ml

filter sterilized (by filtering through a millipore filter) and stored at 0-4°C.

Trace elements: (Gresshoff and Doy 1974) 1000x stock

MnSO\textsubscript{4}.H\textsubscript{2}O : 100 mg
H\textsubscript{3}BO\textsubscript{3} : 30 mg
ZnSO\textsubscript{4} . 7H\textsubscript{2}O : 30 mg
Na\textsubscript{2}MoO\textsubscript{4}.2H\textsubscript{2}O : 2.5 mg
CuSO\textsubscript{4}.5H\textsubscript{2}O : 2.5 mg
CoCl\textsubscript{2} . 6H\textsubscript{2}O : 2.5 mg
Water : 100 ml

dissolved and stored at 0-4°C
Nitrogen-free B₅ stock: (adapted from Gamborg and Eveleigh 1968)

10x stock

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂H₂PO₄·2H₂O</td>
<td>1.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Na₂SO₄·7H₂O</td>
<td>2.5 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>800 ml</td>
</tr>
<tr>
<td>KI</td>
<td>7.5 mg [1 ml of 100x 0.75 g/100 ml stock]</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.5 g (separately dissolved in 199 ml of water and added)</td>
</tr>
</tbody>
</table>

Fahraeus medium: (Vincent 1970) 40x stock

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>4000 mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>4800 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>4000 mg</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>6000 mg</td>
</tr>
<tr>
<td>Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

dissolved and stored in cold

Parasponia nutrient solution: (Trinick 1980) stock (100 ml)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄·7H₂O</td>
<td>7.6 g</td>
</tr>
<tr>
<td>KCl</td>
<td>3.8 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>4.8 g</td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td>2.6 g</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.026 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.058 g</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Na₂B₄O₇·10H₂O</td>
<td>0.344 g</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.025 g</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>0.892 g</td>
</tr>
</tbody>
</table>
Biotin:
For 1000x stock: 10 mg in 100 ml of H₂O
(final concentration 0.001 mg/l)

Thiamine-HCl:
For 1000x stock: 100 mg in 100 ml of H₂O
(final concentration 0.001 mg/l)

2.3.2 Composition of media used for bacteriological studies

Yeast peptone glucose:
Yeast extract 2 g
Peptone 2 g
Glucose 20 g
H₂O 1 litre (pH 6.8)
Glucose is autoclaved separately and added

TY: (Cannon 1980), was used for growth and mutagenesis
Plates
Tryptone 5 g
Yeast extract 5 g
CaCl₂ 0.9 g (pH 6.8)
Agar 15 g
H₂O 1 litre

Liquid
Tryptone 5 g
Yeast extract 3 g
CaCl₂ 0.9 g (pH 6.8)
H₂O 1 litre
Luria broth (Miller 1972)

Yeast extract 5 g
Bacto tryptone 10 g
NaCl 5 g
H₂O 1 litre (final pH 7.0)

For Luria broth with glucose (LBG), glucose was added after autoclaving to a final concentration of 0.5%. Luria broth was used mostly for contamination checks and for storage of E. coli strains.

Bergersen's modified media (BMM) (Bergersen 1969)

<table>
<thead>
<tr>
<th>Components</th>
<th>Recipe (11)</th>
<th>Stocks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>8 ml</td>
<td>45g/l</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>8 ml</td>
<td>10g/l</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>0.15ml</td>
<td>20g/l</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>* Trace elements</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>* Thiamine.HCl</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>* Biotin</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>980 ml</td>
<td></td>
</tr>
<tr>
<td>Na-glutamate</td>
<td>0.5 g</td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 g</td>
<td></td>
</tr>
<tr>
<td>Mannitol (for liquid)</td>
<td>10.0 g</td>
<td></td>
</tr>
<tr>
<td>Mannitol (for plates)</td>
<td>3.0 g</td>
<td>(pH 6.8)</td>
</tr>
<tr>
<td>Agar (plates only)</td>
<td>15.0 g</td>
<td></td>
</tr>
</tbody>
</table>

* Stocks described previously (Section 3.1.1). This was mostly used for growth of Rhizobium strains.
Rhizobium Growth Medium RGM 30 (Mohapatra et al. 1983)

* N-B₅ : 100 ml (see Section 3.1.1 for stocks)
* Trace elements : 1 ml
* Vitamins : 10 ml
* Biotin : 1 ml
* Thiamine·HCl : 1 ml
* Iron chelate : 0.5 ml
Sodium glutamate : 10 mM
H₂O : 785.5 ml pH 6.8

* Stocks described previously, when arabinose was used as carbon source (50mM), it was called RGM30A, if mannitol was used this medium was called RGM30M.

Modified Trifolii Medium with yeast extract (MTMY) (Mohapatra et al. 1983)

* N-B₅ : 100 ml
* Vitamins : 10 ml
* Trace elements : 1 ml
* Fe-chelate : 0.5 ml
* Thiamine : 1 ml
* Biotin : 1 ml
Ammonium sulfate : 0.25 g
Yeast extract : 1.0 g
Arabinose : 4.5 g
Sodium glutamate : 0.75 g
Agar : 15.0 g
H₂O : 887.5 ml pH 6.8

* These stocks are described previously. This medium was used in Tn5 mutagenesis.
2.2.3 **Plant culture media**

**FM:** (for stock solutions see Section 2.2.1)

To prepare one litre of FM, the following were added in order:

- distilled water 500 ml
- \( \text{KH}_2\text{PO}_4 \) 25 ml
- \( \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \) 25 ml
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \) 25 ml
- \( \text{CaCl}_2 \) 25 ml
- Iron chelate 5 ml
- Trace elements 3 ml
- \( \text{H}_2\text{O} \) 392 ml pH 6.5
- agar (plates) 12 g

**Parasponia nutrient solution:** (for stock solutions see Section 2.2.1)

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>mls of stock/l</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CaSO}_4 \cdot 2\text{H}_2\text{O} )</td>
<td>10.0 ml</td>
<td>360 mg</td>
</tr>
<tr>
<td>( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} )</td>
<td>1.5 ml</td>
<td>2.8 ml</td>
</tr>
<tr>
<td>( \text{KCl} )</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>( \text{K}_2\text{HPO}_4 )</td>
<td>3.7 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>( \text{FeNaEDTA} )</td>
<td>2.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>( \text{KNO}_3 )</td>
<td>2.0 ml</td>
<td>500.0 mg</td>
</tr>
<tr>
<td>( \ast \text{CuSO}_4 \cdot 5\text{H}_2\text{O} )</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>( \ast \text{ZnSO}_4 \cdot 7\text{H}_2\text{O} )</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>( \ast \text{CoCl}_2 \cdot 6\text{H}_2\text{O} )</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>( + \text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O} )</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>( + \text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O} )</td>
<td>2.0 ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>( + \text{MnSO}_4 \cdot 4\text{H}_2\text{O} )</td>
<td>2.0 ml</td>
<td>971.0 ml pH 6.8</td>
</tr>
</tbody>
</table>

\( \ast \)Can be combined into a single solution

\( + \) are better added as separate solutions especially \( \text{MnSO}_4 \cdot 4\text{H}_2\text{O} \)
2.2.3 Preparation of antibiotics

Kanamycin, streptomycin and chloramphenicol were used in this project. Each of these antibiotics is water soluble so that the required amount was dissolved in a nominal volume of water, filter sterilized and added to the sterilized medium when cooled to 45°C.

2.3 Bacteriological techniques

Storage

All bacterial strains were stored on BMM (Sm) medium at 0-4°C. Since the cultures of strain ANU289 cannot be stored more than two months, the cultures were subcultured at regular intervals. The Tn5 mutants were stored on TMY-Km-Sm plates.

Incubation

Unless otherwise stated, for growth all incubations were at 28°C. Liquid cultures were incubated in 250ml klett flasks plugged with sterile cotton wool. These were shaken at 180 rpm in gyratory shaker (New Brunswick Scientific Co. Inc. USA). Plates were sealed with a strip of Nesco-film (Nippon Shoji Kaishi, Japan) and incubated inverted. The Tn5 donor E. coli strains were incubated at 37°C for growth.

2.3.1 Bacterial growth measurements

Growth was measured by increase in turbidity using a KLETT-SOMMERSON Photoelectric colorimeter (Klett Manufacturing Co. Inc. New York) using light filter No. 42. The colorimeter scale is graduated in units proportional to optical density (OD); thus Klett values can be converted to O.D. by using the formula

\[
\text{Klett reading} = \frac{1000 \times \text{OD}}{2}
\]
Figure 2.1. Growth curves of strain ANU289 grown in
Bergersen's modified medium (BMM) and RGM30 medium
supplemented with either 10mM arabinose (RGM30A)
or 10mM mannitol (RGM30M) as carbon source (for
composition see Section 2.3.2). Cells were grown in
250ml flasks shaken at 180rpm (28°C)
(i) Nitrogenase activity was monitored in stationary liquid cultures of strain RGM30A (R. meliloti) grown in liquid medium (BMM) at 150 rpm at 30°C. Cells were harvested in early to mid-log phase (about 2.2 x 10^7 viable cells/ml) by centrifugation. The harvested cells were washed once in PBS and then resuspended in 10 mM Tris buffer (pH 7.5) at a final concentration of 10^9 cells/ml. The experiments were carried out in a 100 ml sealed flask (30 ml culture). The flask was purged of air with helium to establish anaerobic conditions. The sealed flask was placed in a temperature-controlled water bath at 30°C. The O_2 concentration was monitored at approximately 3 hr intervals using a CEM/CAN analyzer. A 30 ml sample of culture was taken and mixed with 30 ml of water. The O_2 concentration was monitored and recorded using a Fischer-Schoninger 105 gas chromatograph with a thermal conductivity detector.
(ii) Nitrogenase derepression in stationary liquid cultures:

Cells pregrown in liquid medium (RGM30A being RGM30 plus 10mM D-arabinose) at 180 rpm at 20°C were harvested in mid-late-logarithmic phase (about $2-2.5 \times 10^9$ viable cells ml$^{-1}$). Cells were suspended either directly or after washing (7000xg, 8 min) into derepression medium at a final concentration of $5-6 \times 10^8$ cells ml$^{-1}$. All experiments in liquid cultures were carried out in 27ml scintillation vials (stoppered with size 33 subaseals). The total volume of medium was optimal at 1.0-1.5ml per vial. Vials were evacuated and flushed three times with argon and finally filled with argon 91%, 4% C$_2$H$_2$ and 5% O$_2$. Vials were assayed for acetylene reduction using a Pye-Unicam series 104 or Hewlett Packard 5790A gas chromatograph.

(iii) Derepression of nitrogenase activity in shaken liquid cultures:

Cells pregrown in liquid medium (RGM30M) shaking at 200 rpm at 28°C were harvested in mid-exponential phase (optical density 650nm = 0.13). Cells were washed twice (8000xg, 10 min) in derepression medium (containing succinate 50mM as carbon source and glutamate 3mM as nitrogen source buffered with 50mM MOPS) and diluted to give a final density of about 1-2x10$^9$ cells/ml). Flat bottom Wolfes' bottles (400ml) were used for nitrogenase derepression. Flasks sealed with subaseals (size 33) were evacuated and flushed four times with helium and finally filled with helium, 4% acetylene and oxygen was injected at the described level. The cultures were incubated at 170 rpm and 28°C. The oxygen (0.25%) concentration was monitored at approximately 8 hr intervals by using a GOW-MAC 550 series gas chromatograph with a thermal conductivity detector. Pure oxygen was added to the cultures to maintain the desired oxygen concentration. Samples removed from flasks were assayed for acetylene reduction using a Pye-Unicam series 104 gas chromatograph.
2.3.3 Mutagenesis

(i) Transposon mutagenesis

The suitable selection medium used in crosses was a modified TMY medium (see 2.2.2) containing 30mM D-arabinose plus 4mM monosodium glutamate. Kanamycin (Km) was added to a final concentration of 600 mg/l and streptomycin (Sm) at 500 mg/l. Strain ANU289 (the recipient) was grown for three days on TY slopes at 28°C. Donor *E. coli* strain (transposon donor strain) was grown in LB medium for 16 hrs at 37°C without shaking. Recipient cells were suspended in 3ml of water and plated for viability tests onto BMM medium. Samples (1.5ml) of both donor and recipient suspensions were mixed together and the cells collected on a 0.45 micron millipore filter. Filters containing bacterial crosses were placed onto TMY plates and incubated at 28°C for 24 hrs before the cells were suspended in water. Various dilutions were plated onto selective media and onto LB media. Donor cells plated on LB media were recorded after 24 hours, cross plates were examined and counted after 7 to 10 days of incubation at 28°C. A general scheme of transposon mutagenesis and selection of mutants in strain ANU289 is shown in Fig. 2.2 and the transfer frequencies are shown in Table 2.2.

(ii) NTG mutagenesis

Mid-log phase cultures were washed twice in citrate buffer (pH 5.5) and nitrosoguanidine was added to give a final concentration of 300 mg/l. The mixtures were incubated for 3 hrs at 28°C and the cells were then centrifuged and washed twice in saline phosphate buffer. The final cell pellet was resuspended in original growth medium and shaken for a further 24 hours to allow phenotypic expression and segregation. These mutagenised cells were diluted if necessary and plated onto selective media containing appropriate antibiotics.
Figure 2.2  
**ISOLATION OF MUTANTS USING SUICIDE VECTORS**

**Recipient:**  
*Rhizobium* strain ANU 289 $Sm^R$ pregrown on TY medium slopes

**Donor:**  
*Escherichia coli* 1830 or JMP 575 or ANU 1041 pregrown on Luria broth

- mix on filter
- conjugate for 24 hours at 28°C in a petridish, then select for $Km^R Sm^R$
- test $Km^R Sm^R$ colonies for mutant phenotypes

**Types of mutant:**
- symbiotic
- nod, nif
- ETS
- Hup
- ASM
- auxotrophs

**Selection method**
- nodulation test on plates or cuttings (plate)
- NAD+ test
- tetrazolium chloride test
- growth tests
Table 2.2. Transfer frequencies of transposon Tn5 from different donor strains to strain ANU289

<table>
<thead>
<tr>
<th>donors used</th>
<th>frequency of kanamycin resistant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (1830)</td>
<td>$5 \times 10^{-8}$</td>
</tr>
<tr>
<td>E. coli (JMP575)</td>
<td>$1 \times 10^{-7}$</td>
</tr>
<tr>
<td>E. coli (ANU1041)</td>
<td>$5 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

The method of transposon mutagenesis is described in Section 2.3.3. The frequency of spontaneous $\text{km}^R$ colonies was $10^{-8}$. 
2.3.3.1 Mutant selection procedures

(a) EPS mutants:
NTG mutagenised cells were plated onto RMM plates containing 150mg/l streptomycin. Colonies which appeared to be phenotypically mucoid (identified by a concentric mucoid morphology of larger diameter in contrast to small opaque colonies) were selected and streaked twice on similar plates to purify colonies.

(b) Suc\textsuperscript{−} mutants:
Neomycin resistant colonies after NTG mutagenesis were tested for their ability to grow (a) in presence of the original carbon source and neomycin on which they were selected and (b) alternatively with succinate as the sole carbon source. Colonies which were unable to grow in the presence of succinate alone, but were neomycin resistant were selected and further characterised.

(c) Hup\textsuperscript{−} mutants:
The transconjugants from Tn5 mutagenesis were plated onto TMY plates containing TTC (0.1g/l) and kanamycin-streptomycin to select for transconjugants defective in Hup phenotype. Colonies which did not reduce the dye or exhibited low dye reduction ability after 10 days were selected and restreaked onto TTC containing medium. TTC when reduced turned red meaning the transconjugant was Hup\textsuperscript{+}.

(d) Ets mutants:
Mutant colonies on plates either from NTG or transposon mutagenesis, when grown up to 1mm in diameter, were subjected to an oxidase test. Selection at two different levels are described below.
Primary screening: Plates growing mutants were flooded with a freshly prepared 1:1 mixture of 1% alpha-napthol in 95% ethanol and 1% N,N-dimethyl-p-phenelene diamine sulphate or oxalate in water. After 15 to 30 sec exposure to the reagent the plates were drained completely. Colonies with functional terminal oxidases stain a deep blue colour within a few minutes. Colonies with possible altered staining characteristic were restreaked onto fresh antibiotic plates within a few minutes after application of the stain. These colonies were later rechecked for growth on LB(LB+ colonies, if any, were discarded) and nodulating ability on siratro.

Secondary screening: Both the wild type and possible mutants selected after primary screening were grown to mid-log phase in BMM. The cultures (10 ml) in duplicate were centrifuged (to remove most of the exopolysaccharide) at3000xg for 10 min and the pellet resuspended in equal volume of phosphate buffer (pH 6.8). Then 0.05 ml of 1% DMPD in water and 1% alpha-napthol in 95% alcohol were separately added to the cells in test tubes. These tubes were then sealed, evacuated and filled with 100% oxygen using an Autosuk gas exchange device. The tubes were shaken at 225 rpm for 30 min at 28°C. The cells were then centrifuged at 8000 rpm for 10 min and the supernatant was assayed at 750 nm. Phosphate buffer alone served as a control.

(e) Enrichment strategy and selection of ammonium assimilation defective (Asm−) mutants

To select for transconjugants which may be defective in ammonium assimilation, the bacteria from the filter were resuspended into RGM30A containing 5mM each of glutamine and glutamate (freshly prepared; filter
sterilized and added) and the required antibiotics. These cells were allowed to shake for 12 hrs, were spun and resuspended in N-RGM30A for 3 hours followed by the addition of NH₄Cl (10mM) and penicillin G (Sigma, 5mg/ml) and incubated shaking for 24 hrs. After two washes with sterile water, these cells were resuspended in 5mm each of glutamine and glutamate and shaken for 24 hours. Samples (0.1ml) of this were plated onto media containing gluconate (5g/l, as carbon source), (NH₄)₂SO₄ (1g/l, as nitrogen source) and the antibiotics. The slow-growing colonies (which grow by scavenging nitrogen from agar) on these plates were presumably unable to use ammonium sulphate as the sole nitrogen source. These were chosen for growth tests in liquid media containing either ammonium sulphate, glutamine or glutamate as the nitrogen source and gluconate as the carbon source. Gluconate without any nitrogen source did not support growth.

2.3.4 Other Assays

(i) Acetylene Reduction assay

Nitrogenase activity was measured by using acetylene reduction assay (Dilworth 1966) in which nitrogenase converts acetylene to ethylene. Both gases can be quantitatively measured by gas chromatography.

Acetylene gas was always freshly prepared by adding a few chips of calcium carbide to distilled water in a specially designed apparatus (see Burris 1974). The gas generated at one atmospheric pressure (by upward displacement of water) was (relatively pure, contained only trace amounts of CH₄ and C₂H₄) compared to commercially available compressed gas. The acetylene was removed from the reaction flask using a syringe and injected into the assay vials.
Figure 2.3. Calibration of integrator units to ethylene concentration: a graphic comparison of the calibration curve (●) and its fitted model (○). Each point on the calibration curve represents the average integrator value for a specific ethylene concentration.
A "Pye-series" line of a Hewlett-Packard 3390A gas chromatograph was used to measure the ethylene and ethane in each sample. The gases in each sample were passed through a Pye C 100-100 m Yukawa glass at 100°C and were detected by a flame-ionisation detector. The ethylene was integrated to a numerical value by a digital integrator (Hewlett-Packard 3390A, integrator-reference). Using a dilution series of ethylene (4.00 ± 0.05 nmoles) on ethylene integrator units, a calibration curve was obtained (Fig. 2.3). The elimination of the time-consuming process of reading values was eliminated. The following equation was fitted using the least squares method to the experimental data for the calibration curve:  

\[ y = \frac{ax^b}{1 + cx^b} \]

where \( y \) is the number of ethylene integrator units, \( x \) is the calibration value in nmoles of ethylene, \( a, b, c \) are constants, and \( x \) is the dilution factor. The constant pair \( a = 1.02, b = 1.00 \) was used (Wright, 1988) and is shown in Fig. 2.3. The fitted equation was then used on a Hewlett-Packard 25 calculator for an immediate conversion from ethylene integrator units to moles of ethylene.

The cultures were irradiated under a dimness containing 0.12-0.17% ethylene in the gas mixture.

Samples of 10 ml of each at were collected 0 min following the onset of ethylene reduction. Ethylene gas of 20 ml at rate 50 ml/sec was added to the culture, whereas in 40 ml of "Phytosan" gas was used. By deleting culture experimental condition, the sample was used.
A Pye-Series 104 or a Hewlett Packard 5790A gas chromatograph was used to measure the ethylene and acetylene in each sample. The gases in each sample moved through a Poropak R (80-100 mesh) column at 55°C and were detected by flame-ionization at about 60 sec. The amount of each component gas was integrated to a numerical value by a digital integrator (Autolab 6300) or Hewlett Packard 3390A integrator-recorder.

Using a dilution series of ethylene (CIG, Australia) an ethylene-integrator units calibration curve was obtained (Fig. 2.3). To eliminate the time consuming process of reading values of ethylene off the calibration curve, the following equation was fitted using the statistically-oriented computer language GLIM (Nelder 1975):

\[ E = 0.8458 + (1.088 \times 10^{-3})U + (1.635 \times 10^{-9})U^2 \]

where \( E \) = the number of nmoles of ethylene in the incubation vial; and

\( U \) = the units recorded by the integrator.

A comparison of the fitted equation to the actual calibration curve was made (Carroll 1980) and is shown in Fig. 2.3. The fitted equation was programmed to a Hewlett Packard 25 calculator for on the spot conversion of integrator units to nmoles of ethylene.

Nitrogen fixation was measured in derepressed cultures, detached nodules and whole plants using this acetylene reduction assay. The assay conditions are described below.

(a) Acetylene reduction in derepressed cultures

The cultures were incubated under a gasphase containing 4 to 5% acetylene in the gas phase.

Samples of 0.25 or 0.5 ml were withdrawn depending on the volume of experimental flask. From 27 ml vials usually 0.25ml, samples were withdrawn, whereas in 400 ml flasks (as was used in shake culture experiments) the sample size was 0.5 ml.
(b) Acetylene reduction in whole siratro plants

The plants were removed from agar plates and placed into scintillation vials along with a few drops of distilled water. The vials were sealed and filled with 10% acetylene. These were incubated at 25°C under a photon flux density of approximately 220 μEm⁻² s⁻¹ throughout the experimental period. Gas samples were withdrawn at regular intervals over a period of 6 hrs and analysed by gas chromatography. Six plants were examined for each treatment.

(c) Acetylene reduction in detached nodules

Parasponia and siratro plants inoculated with Rhizobium strain ANU289 were grown in sand with a nitrogen-free nutrient solution at 24 to 28°C with light intensity of about 350 μEm²s⁻¹ during daylight period. Nodules were detached from plants of siratro (10 week old) and Parasponia (8 weeks old) and were immediately placed into experimental vials with 0.1 ml of sterile water (enough to wet the flask, but not to submerge the nodule). Nodule sizes were about equal in mass though Parasponia nodules were cylindrical, whereas siratro nodules were spherical. These vials were evacuated and flushed twice with argon and filled with argon, 10% C₂H₂ and the desired oxygen concentration. Acetylene reduction was measured after 60 and 120 min.

(ii) Assay for exopolysaccharides:

Extracellular polysaccharides were prepared by placing liquid cultures in a boiling water bath for 15 minutes (Kang and Markovitz 1967). This treatment released essentially all capsular polysaccharides from the cells. Cells were removed by centrifugation at 5000xg for 10 min followed by second centrifugation at 12000xg for 20 min. The supernatant was
dialysed overnight against distilled water (about 1 litre) with five changes. EPS was measured as glucose using the modified anthrone method (Shields and Burnett 1960). Portions (2 ml) of anthrone solution were added dropwise to 1 ml of the supernatant previously cooled to under 4°C by immersion in an ice bath for 45 min. After mixing the contents the firmly stoppered tubes were immersed in a water bath at 92°C for 8 min. On withdrawal the tubes were reimmersed in an ice bath to stop the reaction. The absorbances (at 630 nm) were read after 30 min in a Pye-Unicam SP30UV spectrophotometer. All estimates were done in duplicate.

(iii) Measurement of oxygen consumption

Oxygen consumption was measured both in aerobically grown cells and microaerobic nitrogen-fixing cultures.

Measurements of O₂ consumption rates in aerobic suspension cultures were made with a Clark-type oxygen electrode at the bottom of a cylindrical water jacketed, magnetically stirred chamber (Rank Bros. Bottisham, Cambridge, U.K.). The electrode was calibrated injecting a small measured volume of air saturated water (at Canberra, solubility of oxygen being 76 mg/l). The current was reliably measured for dissolved oxygen concentrations in the range of 1µM to 0.3mM. A strip chart recorder with variable chart speed was used. The readings were usually taken during logarithmic growth phase and were done in duplicates.

In nitrogen-fixing vials, the oxygen consumption was measured by determining oxygen in the gas phase using a GOW-MAC 552 gas chromatograph. A molecular sieve (mesh range 60-80) 5A column (at 70°C) coupled to a thermal conductivity detector (at 200°C) was used. A Hewlett Packard 3390A plotter-integrator converted the components of sample into numerical units. Oxygen concentration in the gas phase was monitored by comparing
the values with a calibration curve prepared out of a dilution series of pure oxygen. The syringe was flushed 8-10 times with carrier gas before samples containing oxygen were measured. Oxygen consumption was measured at a regular interval during the nitrogen-fixing period. The consumption was calculated by subtracting the remaining from the initial oxygen in the gas phase.

(iv) Measurement of hydrogen uptake/evolution

A GOW MAC 552 gas chromatograph equipped with a thermal conductivity detector set at column temperature (60°C), injection temperature (100°C), cell temperature (100°C) and 80mA current was used for measurement of hydrogen. A 6.4mm diameter by 1.8m column of molecular sieve 5A (60-80 mesh), and a flow rate of 30 ml/min of carrier gas (argon) was used. Gas samples containing H₂ were injected into the gas chromatograph by a syringe and the readings were quantitatively expressed by comparing to the calibration curve prepared by a dilution series of pure hydrogen.

(v) Ammonium determination

Ammonium was determined using Phenol-calcium hypochlorite method of Gresshoff (1981). The calcium hypochlorite stock solutions were prepared by stirring 25g CaCl₂ in 300ml of hot water, until it dissolved. To this was added 135ml of K₂CO₃ solution (heated if necessary). The final volume was finally diluted to 500ml. The mixture was filtered and stored in cold.

The assay was performed by adding 0.5ml of phenol (from a stock of 20% crystalline phenol in ethanol), 1ml of sample or standard and 1ml of
calcium hypochlorite stock. The reaction mixture was transferred to a cuvette and read against blank at 650nm using a SP30UV Spectrophotometer. The concentration of ammonia was determined from an ammonium chloride standard curve.

(vi) Glutaminase assay

Cells were grown in RGM30M with glutamate or glutamine as nitrogen source (5mM). The cells were harvested at late-log phase, centrifuged at 8000xg for 10 min and washed in potassium phosphate buffer (pH 7.2). The supernatant was saved while the pellet was resuspended in 10 ml of potassium phosphate (pH 7.2) and sonicated (Labsonic 1510), for 2 min (30 sec bursts) at 4°C at 100W. Cell debris was pelleted by centrifugation for 60 min at 4°C at 10,000xg; the supernatant fluid constituted the crude cell extract. This was dialysed overnight against potassium phosphate buffer. The dialysate was used for enzyme assays.

Glutaminase was assayed by determining ammonium released in the presence and absence of L-glutamine. The ammonium determination procedures was described above. The samples from both supernatant and crude extract were incubated at 37°C with or without glutamine. One ml samples were withdrawn from these incubation at 20 min intervals and assayed for ammonium by the phenol-hypochlorite method (Gresshoff 1981). The enzyme activity was expressed as µmoles min⁻¹ mg⁻¹ protein.

(vii) Glutamine uptake assay

Strain ANU289 cells were grown in 100ml of RGM30M containing glutamate or glutamine as a sole nitrogen source (freshly prepared glutamine was added to the growing culture at regular intervals). The cells were harvested at early-log phase, washed in nitrogen-free RGM30M medium and finally resuspended in 10 ml of the same medium.
Radioactive solute and glutamine and/or glutamate in nitrogen-free medium were added to 0.4 ml of cells (at desired concentrations) to give a final volume of 1.0 ml. The reaction was started by adding cells to the nitrogen-free medium with appropriate concentrations of glutamine and glutamate. After 5 min incubation (by shaking) the radioactive glutamine (Amersham, U.K.) was added and the shaking was continued for 17 min. The contents of these vials were then placed on a millipore filter attached to an Amicon Vacuum Manifold. The filter was washed three times with washing buffer (nitrogen-free RGM30M) and put into a scintillation vial containing 0.75 ml of 2-methoxyethanol. To this was added 0.2 ml of H₂O and 10 ml of scintillation liquid (40 ml of 25x Permafluor plus 500 ml of Triton x and 1000 ml of toluene). A microprocessor controlled Beckman LS 7000 liquid scintillation system was used for the counting.

2.3.5 Large scale bacteroid isolation

Bacteroids were prepared from nodules by grinding with chilled mortar and pestle in a buffer containing 0.05M potassium-phosphate, 0.3M sucrose, 5mM MgCl₂ and 0.2M Na-ascorbate (pH 7.5). After washing twice in the cold grinding buffer, 10g nodules were hand ground in 10 ml of grinding buffer and 3g Polyclar AT (insoluble PVP, General Aniline and Film Corporation N.Y., acid washed and grinding buffer saturated). The homogenate was filtered through 2 layers of mira-cloth and the filtrate centrifuged at 200xg for 10 min to remove starch granules, nuclei and aggregates. The post nuclei supernatant was layered on 10 ml of 35% (w/v) sucrose in 50 mM potassium-phosphate buffer (pH 7.5), centrifuged for 15 min at 17000xg and the pellet resuspended in 10 ml of the original buffer. Five ml each were layered on stepwise gradients prepared in 50 mM phosphate buffer consisting of 8 ml of 45%, 10 ml of 50%, 8 ml of 52% and 5 ml of 57%
sucrose (w/w). All the gradients were centrifuged for 4.25 hr at 90,000xg in a Beckman SW27 rotor. The gradient was displaced with a 65% (w/w) sucrose solution and fractionated. One ml fractions were collected. Optical density was measured at 680nm and specific gravity was measured by a Bausch and Lomb refractometer.

2.3.6 Method of cytochrome analysis in cultured cells and bacteroids

Preparation of cell-free extract

Rhizobium strain ANU289 was grown with shaking at 200 rpm in 41 flasks containing 21 of TY medium. After 8 days of growth, the cultures were harvested and washed with 0.1M phosphate buffer (pH 6.8) and resuspended to 25% (wet w/v) in the same buffer. In case of bacteroid preparations the bacteria from individual interfaces 45 - 50%, 50 - 52% and 52 - 57% were harvested separately, washed with 50mM phosphate buffer pH 7.5 and finally resuspended in 0.1M phosphate buffer pH 6.8.

Crystalline deoxyribonuclease (2 mg/l) and MgCl₂ (2mM) were added in the bacterial suspension and the mixture passed twice through a French Pressure Cell. The cellular debris was separated by centrifugation at 12,000xg for 30 min. Recentrifugation at 187,000 xg (90 min) of the supernatant gave a translucent amber pellet and a clear red-brown or amber supernatant. The pellet fraction was washed with 0.1M phosphate (pH 6.8) and resuspended to 25% (wet w/v) in the same buffer and used as the membrane fraction. The supernatant was recentrifuged to remove any remaining membrane fraction. A summary of methods used for the preparation of bacteroids, preparation of particles and spectral analysis for cytochromes is presented (Fig. 8.2).
Cytochrome spectra

Essentially the method of El Mokadem and Keister (1982) and Keister et al. (1983) was followed. Cytochrome spectra for the membrane fraction or supernatant were first determined in respect of difference spectra of sodium dithionite reduced minus air or ferricyanide oxidised preparations. Carbon monoxide difference spectra were obtained by equilibrating the dithionite reduced samples with 10 vol of pure CO through a syringe for 15 min followed by 15 min further equilibrations in closed cuvettes. 1.0 cm light path cuvettes were used in Hitachi Perkin Elmer Model 557 spectrophotometer to obtain these spectra. In cultured bacteria the low temperature spectra were taken in 2mm cuvettes at liquid nitrogen temperature.

The approximate concentration of each cytochrome and CO-reactive pigment of both particulate and supernatant fraction was calculated from reduced-minus-oxidised or reduced plus CO-minus-reduced difference spectra respectively, by measuring the difference between a wavelength pair corresponding to an absorption maximum (or peak) and absorption minima (trough or plateau) of that component or related component in pure solution. Extinction co-efficients were those used by Appleby (1969 a, b) and Smith (1978) and are as follows:

<table>
<thead>
<tr>
<th>Extinction Coefficients</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt a</td>
<td>13.1</td>
</tr>
<tr>
<td>cyt a-CO</td>
<td>10.1</td>
</tr>
<tr>
<td>cyt b</td>
<td>17.9</td>
</tr>
<tr>
<td>cyt c-CO</td>
<td>6.54</td>
</tr>
<tr>
<td>cyt c</td>
<td>23.2</td>
</tr>
<tr>
<td>cyt c-CO</td>
<td>17.6</td>
</tr>
<tr>
<td>cyt P420-CO</td>
<td>111.0</td>
</tr>
</tbody>
</table>
2.3.7 Protein determination

All protein determinations were made using a modified Lowry procedure adopted from Schacterle and Pollack (1973). Bovine serum albumin was employed as protein standard. If necessary samples were diluted with phosphate buffer.

2.3.8 Contamination tests

Microbiological purity of inoculant cells and original media in assay vials after completion of the experiment was tested on LBG [Luria broth plus glucose (Miller 1972)] and BMM medium and antibiotic resistance of single colony isolates. Nodulation test (using axenic plant culture techniques) on Fahraeus medium (Rolfe et al. 1980) was used to characterise representative single colony isolates. Bacteroids isolated from surface-sterilised nodules of test plants confirmed the morphological, genetic and biochemical characteristics of all tested strains.

2.4 Plant culture methods

Siratro

To meet the requirement of the large quantity of nodules, siratro was planted in pots (6" in diameter) on sterilized coarse sand or sand-vermiculite mixture. Siratro seeds were sterilized (see Section 2.4.1) and germinated on FM plates. After 12-18 hrs the seedlings were inoculated with the appropriate Rhizobium strain. These plates were incubated in a growth cabinet with light at 28°C for further 24 hours. About 7 to 10 seedlings were planted per pot. The pots were inoculated further with the same Rhizobium culture at 2d interval for one week and were irrigated with a 'quarter strength' FM twice a week. The nodules were ready for harvest in about 6 to 8 weeks.
Figure 2.4. Parasponia propagation by stem-cutting method. (A)

A healthy plant is chosen to collect stem-cuttings. (B)

A growing branch is cut from the plant. The cuttings are chosen such that each of them contains at least one node, a few buds and/or leaves. These are treated with indolebutyric acid (IBA) for 5 min and planted into pots of 6 cm diameter (C) or cotton blocks on a tray (D). These pots and cotton blocks are kept under a spray mist until roots have appeared. The stem cuttings with roots are then transferred singly to larger pots (15 cm diameter) containing pot-mix. (F) These Parasponia plants are maintained in a glass house at 20-28°C with a photon flux of about 350 µE m⁻² s⁻¹.
Parasponia

*Parasponia rigida* used in this study was dioecious, thus from 2 plants initially germinated, it was not possible to obtain seeds. Besides the seeds were reported to take considerable time (4-6 weeks) to germinate. Therefore, the propagation of *Parasponia* plants was done by stem-cuttings. The clonal cuttings were induced to root by treating the cut-surface with indole-butyric acid. These cuttings were kept under spray mist in the greenhouse. After sufficient roots had developed (usually 2-3 weeks), the plantlets were transferred into bigger pots (6" diameter). The pots or cuttings were inoculated with excess *Rhizobium* and were placed either in a growth chamber (28°C) or glasshouse. The nodules were ready for harvesting in about 10 to 14 weeks, although it was possible to show that under optimal conditions, nitrogen-fixing nodules could be obtained within 6-7 weeks after the initial removal of the cutting. Figure 2.4 illustrates the techniques of *Parasponia* propagation by stem-cutting.

2.4.1 Nodulation tests

(a) *Siratro*

(i) Sterilization of seeds

One scoop (about 1 g) of calcium hypochlorite powder was vigorously stirred into about 50 ml of water, then allowed to saturate at room temperature for about 1 hr.

Siratro seeds were submerged in concentrated H₂SO₄ for 20 min, with shaking periods. The seeds were then washed three times in water and then transferred to a petridish and washed with alcohol to remove water. These seeds were treated with saturated calcium hypochlorite for 15 min, and washed 5 times with sterile distilled water and placed onto FM agar plates (about 80 seeds per plate). The plates were allowed to sit on the flowhood for one hour and then incubated upright in a dark 28°C incubator for 12-18 hours.
NODULATION TEST WITH PARASPONIA — RHIZOBIUM STRAIN ANU 289

**SIRATRO**
- Treat seeds with $\text{H}_2\text{SO}_4$ for 20 mins
- Surface sterilise with calcium hypochlorite for 10 mins
- Place sterile seeds on FM agar plate
- Germinate seeds in dark for 24 hours at 30°C
- Select uniform and healthy seedlings and place two seedlings onto a Rhizobium pre-inoculated, nitrogen free (FM medium)
- Incubate vertically at 28°C, 16hr photoperiod of 350 μEm$^{-2}$
- Score for symbiotic characters and plant performance (6—8 weeks)

**PARASPONIA**
- Potted parent plant
- Rooted clonal cutting (IBA induced, greenhouse spray misted)
- Inoculate with excess Rhizobium
- Nodulated cutting
- Grown in greenhouse or growth chamber (28°C)
- Score symbiotic characters and plant performance (10—14 weeks)
Figure 2.6. Illustration of nodulation tests of strain ANU289 with the non-legume *Parasponia* (A₁ - A₃) and the tropical legume siratro (B). A₁ - *Parasponia* stem-cuttings treated with IBA (see Figure 2.4) are put onto sterilized cotton blocks contained in separate 6 x 6 cm pots. These blocks are inoculated with strain to be tested and kept under spray mist until the roots have appeared. A₂ - Then these cotton blocks are transferred to individual pots of sand plus pot-mix. A₃ - After 10-14 weeks the nodules are harvested and used for nitrogen fixation and other tests. (B) Nodulation tests on siratro are done using the procedure of Rolfe et al. (1980) and is described in Section 2.4.1.
(ii) Plate method

Fahraeus medium plates were inoculated with two drops of a 1:2 dilution in sterile distilled water of the appropriate inoculant strain, and was spread with a glass spreader without touching the sides of the plate. Plates were stored at 0-4°C and if dry, sterile distilled water was added. Two germinated seedlings (root length not greater than 2cm) were placed onto a line 2/3 the plate. The plates were left in a horizontal position for about 60 min. These were then sealed with Nesco-film making sure that the lower half of the seal was complete (to prevent leakage) and that a 1-2 cm long slit was introduced along the top section (to allow gas exchange). The plates were incubated in a growth chamber at 16h light -8hr dark photoperiod (with a photon flux of about 300 µEm⁻²S⁻¹) at 28°C. The root system was screened from excessive light exposure by stacking the plates in rows taking care that the outer plates were sealed appropriately (e.g. by cardboard cards or uninoculated agar plates). These plates were checked at regular intervals and when dry, sterile distilled water was injected through the slit. Nodulation was clearly seen by 14 days and clear growth differences were visible by 30 days. Uninoculated controls were always included.

(b) Parasponia

Although at present an improved method of nodulation test is being used, a slightly different method was followed during the tenure of this investigation. The cubical cotton blocks were sterilized and soaked with a diluted bacterial inoculum before the leaves or stem-cuttings (treated with indole-butyric acid) were planted onto them. About 10ml of inoculum was supplied to each of the cotton block at weekly intervals. Bacteria isolated from these nodules were checked for inoculant markers. A comparative diagram of nodulation tests in siratro and Parasponia is
presented (Fig. 2.5 and Fig. 2.6). Using this method it was shown that 10 bacterial isolates each (randomly chosen) out of 10 different Parasponia nodules (i.e. 100 isolates) originally inoculated with ANU289 still retained the genetic markers of ANU289 (Gresshoff, unpublished data).

2.4.2 Reisolation of *Rhizobium* from root nodules (based on Rolfe and Gresshoff 1980)

Nodules were excised leaving a small length of the attached root. The nodules were transferred to a small test tube of distilled water and vortexed to wash. These were dipped in 95% ethanol for one minute with 10% (v/v) hypochlorite containing 0.2M mannitol and rewashed in a test tube with sterile distilled water by repeated vortexing. These nodules were checked for their surface sterility by a rolling test on both LB and BMM plates. The nodules were finally squashed in phosphate buffer (0.1M KH$_2$PO$_4$, pH 7.2) containing 0.2M mannitol. The squashate was then serially diluted and plated onto high mannitol (39g/l) BMM with appropriate antibiotics [usually streptomycin (150mg/l) and in the case of transposon induced mutants kanamycin (500 mg/l)].

2.4.3 Microscopy

The light and electron microscopy work was done in collaboration with Mr Dean Price (Botany Department, ANU). Fractionated bacteroids of siratro and Parasponia were scanned using the facilities and with the help of Dr H Calvert (C.F. Kettering Research Laboratory, Yellow Springs, Ohio).

The routine fixation and embedding procedure was used. About 1-2mm of tissue pieces from the excised nodule was placed into a vial of formaldehyde-glutaraldehyde fixative (overnight at room temperature).
The fixative was removed and the pieces were washed twice with 0.025M phosphate buffer (pH 7.0) and then post-fixed with 1% OsO₄ in phosphate buffer for 1-2 hr at 0°C. The tissue was rinsed in phosphate buffer and was dehydrated for 30 min in each of 30%, 50%, 70% and 90% acetone and for 3 ten-minute changes in 100% acetone. The tissue was infiltrated by adding Spurrs resin at various intervals such that the mixture was 1:2 Spurrs resin:acetone for 1 hr, 1:1 Spurrs resin:acetone for 1 hr, 2:1 Spurrs resin:acetone for 1 hr and finally 100% Spurrs resin for 1 hr. Fresh resin was then added to fully impregnate the tissue overnight. The tissue was removed from the vial and put in a capsule half-filled with polymerised resin, and more fresh resin was added thus embedding the specimen in the middle of the capsule. The mould was placed in the oven for 24 hrs. The specimen was cut from the block using a small saw, then mounted in a microtome and trimmed. Sections were cut with a glass or a diamond knife for light and electron microscopy, respectively.

For scanning electron microscopy, the bacteroid samples from different fractions were first washed and then passed through a 0.6μm nucleopore filter. The filter was treated using the routine fixation procedure and scanned.

2.4.4 Partial purification of leghemoglobin

Siratro: The supernatant from 35% (w/v) sucrose centrifugation was recentrifuged at 17,000xg for 30 min and the supernatant thereof was fractionated with solid ammonium sulphate with the pH maintained between 6.5 - 7.0. The fraction precipitating between 47 and 80% saturation was re-dissolved in a small volume of the 20mM phosphate buffer (pH 6.8), dialysed against 1mM EDTA (pH 6.8) and concentrated by pressure filtration over a Diaflo PM10 membrane. This concentration was then passed through a Sephadex G25 column. The elute from the G25 column was fractioned on a
27cm x 2cm column of Whatman DEAE - cellulose in acetate buffer (pH 5.6) which was pre-equilibrated with 0.015M ammonium acetate over a linear gradient of acetate buffer (0.015M, pH 5.6 to 0.151 pH 5.6). The effluent was collected in 2 ml fractions with a LKB Rati Rac fraction collector (LKB Produkt, Stockholm). The fractions were analysed for absorbance at 540nm and the major fractions were used for electrophoresis and absorption spectra analysis.

**Parasponia**: In the case of Parasponia nodules, a modified grinding buffer containing 0.1M phosphate buffer pH 6.8, 0.3M sucrose, 5mM MgCl₂, 5mM sodium ascorbate and 2mM EDTA was used. The samples for gel electrophoresis were prepared by ammonium sulphate fractionation followed by ultra-filtration. Alternatively, the supernatant was concentrated by a Diaflo PM10 membrane during which samples were removed, centrifuged and supernatant was used for spectral analysis and gel electrophoresis.

In anaerobic preparations the above grinding buffer was used. The nodules frozen in liquid N₂ were dropped into the steel chamber of an Omnimixer (Sorvall) containing two volumes of extraction buffer, 0.3g of Polyclar AT per gram fresh weight of nodules. The mixture was ground at full speed under argon for 2 min at 20°C, then centrifuged under argon at 17000xg for 1 hr at 0°C. The supernatant stored under argon was used for SDS-PAGE electrophoresis.

2.4.5 Electrophoresis

A Bio Rad Model 220 dual vertical slab gel electrophoresis cell (BIORAD Laboratories, Richmond, CA) was used for SDS-PAGE disc electrophoresis.
I  **Separation gel (24 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>for 12.5% gels</td>
<td>9 ml</td>
<td>1.0 M Tris HCl pH 8.8</td>
</tr>
<tr>
<td></td>
<td>10 ml</td>
<td>30% Acrylamide stock</td>
</tr>
<tr>
<td></td>
<td>0.6 ml</td>
<td>4% SDS</td>
</tr>
<tr>
<td></td>
<td>2 ml</td>
<td>H$_2$O</td>
</tr>
<tr>
<td></td>
<td>2.4 ml</td>
<td>15 mg/ml ammonium persulfate</td>
</tr>
<tr>
<td></td>
<td>6 µl</td>
<td>TEMED (degassed for 45 sec before add TEMED)</td>
</tr>
</tbody>
</table>

II  **Stacking gel (20 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ml</td>
<td>0.25 M Tris HCl, pH 6.8</td>
</tr>
<tr>
<td></td>
<td>2.0 ml</td>
<td>Acrylamide stock 30%</td>
</tr>
<tr>
<td></td>
<td>0.6 ml</td>
<td>4% SDS</td>
</tr>
<tr>
<td></td>
<td>5.8 ml</td>
<td>H$_2$O</td>
</tr>
<tr>
<td></td>
<td>2.0 ml</td>
<td>15 mg/ml ammonium persulfate</td>
</tr>
<tr>
<td></td>
<td>0.005 ml</td>
<td>TEMED</td>
</tr>
</tbody>
</table>

H$_2$O was removed from separation gels; the stacking gel was then loaded up to 1 cm of height, and water was overlayed on the stacking gel. Gels were stored overnight in humidified plastic bags at 10°C.

III  **Sample buffer**

(20 ml, 2x conc) 10 ml 0.25 M Tris HCl pH 6.8  
(final conc 0.0625M)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Details</th>
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<tbody>
<tr>
<td></td>
<td>4.0 ml</td>
<td>glycerol 10%</td>
</tr>
<tr>
<td></td>
<td>0.8 ml</td>
<td>0.05% Bromophenol blue 0.001%</td>
</tr>
<tr>
<td></td>
<td>0.8 g</td>
<td>SDS 2%</td>
</tr>
<tr>
<td></td>
<td>5.2 ml</td>
<td>H$_2$O</td>
</tr>
</tbody>
</table>

(Store at room temperature)
IV Sample preparation

(adj usted volume of $H_2O$ and protein as needed)

e.g. 0.1 ml sample buffer (III above)

0.05 ml sample

0.04 ml of $H_2O$

0.01 ml of 2-mercaptoethanol

Mercaptoethanol was added and mixed to the sample prior to a run, heated for 5 min in boiling water and an appropriate amount of the sample was applied.

The gels were run at 3 mA/gel until the marker reached the bottom of the gel after 3-5 hrs, fixed in 50% TCA overnight (at room temperature), stained for 1 hr at 37°C in 0.1% Coomassie Brilliant Blue (R-250) in 50% TCA (stored at 4°C), and finally destained by washing repeatedly in 7% acetic acid.

V List of solutions

1. Electrode buffer: 0.025M Tris base (3.02 g/l)

   0.192M Glycine (14.41 g/l)

   0.1% SDS (1 g/l)

   adjusted pH 8.3 with a few drops 1M HCl if needed.

2. Acrylamide stock: 30% Acrylamide

   0.8% N,N'-bis-methylene acrylamide

VI Molecular weight markers

The low molecular weight marker proteins (Fig. 2.7) used in this study included the following:
Figure 2.7. Relative mobility of low molecular weight marker proteins on SDS-PAGE (12.5% gel). The marker proteins used were albumin (Bovine plasma, 66000) albumin (egg ovalbumin, 45000), trypsinogen (Bovine pancreas PMSF treated 24,000), β lactoglobulin (Bovine milk 18400) and lysozyme (egg white 14,300).
bovine albumin
egg albumin
tryptsinogen
β-lactoglobulin
lysozyme

Mw x 10000

relative mobility

0 0.5 1.0
<table>
<thead>
<tr>
<th>Protein Type</th>
<th>MW (daltons)</th>
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</thead>
<tbody>
<tr>
<td>Albumin (Bovine plasma)</td>
<td>66,000</td>
</tr>
<tr>
<td>Albumin (Ovalbumin, egg)</td>
<td>45,000</td>
</tr>
<tr>
<td>Trypsinogen (Bovine pancreas) PMSF treated</td>
<td>24,000</td>
</tr>
<tr>
<td>β-Lactoglobulin (Bovine milk)</td>
<td>18,400</td>
</tr>
<tr>
<td>Lysozyme (Egg white)</td>
<td>14,300</td>
</tr>
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</table>
CHAPTER-3

DEMONSTRATION OF IN VITRO NITROGENASE ACTIVITY IN ASYMBIOTIC CULTURES OF RHIZOBIUM STRAIN ANU289
3. DEMONSTRATING OF IN VITRO NITROGENASE ACTIVITY IN ASYMBIOTIC CULTURES OF RHIZOBIUM STRAIN ANU289

3.1 Background

Until recently, the control mechanisms governing symbiotic nitrogen fixation were studied using either excised root cultures (Lewis and McCoy 1933, Raggio et al. 1957), isolated bacteroids (Bergersen 1975), detached nodules (Sutton and Jepson 1975), or nodule protoplasts (Schetter and Hess 1977). The expression of the enzyme nitrogenase, catalysing the reduction of atmospheric dinitrogen to ammonia, was considered to be confined to Rhizobium within the nodule. This was because of the inability to demonstrate the nitrogen-fixing capacity of Rhizobium in axenic vegetative cultures.

Attempts to show nitrogen fixation with free-living legume bacteria were first documented by Virtanen et al. (1947). The emphasis, however, in these experiments was to demonstrate the indispensibility of leghemoglobin to nitrogen fixation in root nodules, not realizing the significance of asymbiotic fixation. The results were not conclusive due to inconsistency in replicate experiments and the lack of highly sensitive methods such as acetylene reduction (Dilworth 1966), and incorporation and enrichment (Burris and Wilson 1957).

Dunican and Tierney's (1974) transfer of genetic material from R. trifolii to non-nitrogen fixing Klebsiella aerogenes also failed to provide direct evidence for the occurrence of the nitrogenase genes in R. trifolii. Later, the presence of an electron transport factor associated with nitrogenase and a protein immunologically similar to the Mo-Fe protein component was demonstrated in cultured cells of R. japonicum (Bishop et al. 1975). However, nitrogenase was not functional here, because only one of the two components was present. Evidently the physiological conditions required for nitrogenase activity were not fulfilled; additionally more recent work has shown that in R. japonicum nifH and nifDK are not closely linked, and therefore may be genetically controlled to varying degrees of stringency (Hennecke 1983, Shaw 1983).
Reports in 1975 on in vitro symbiosis between plant cells and Rhizobium (see Appendix 1) provided the primary impetus for efforts culminating in the discovery of nitrogen fixation in free-living Rhizobium. Simultaneously three different laboratories (Kurz and LaRue 1974, McComb et al. 1975, Pagan et al. 1975) demonstrated nitrogenase activity (as determined by acetylene reduction as well as $^{15}$N incorporation in some cases) in several Rhizobium strains cultured on agar slopes. Most of these strains belonged to the 'cowpea miscellany' and R. japonicum groups. Optimal activity was found in a media containing two carbon sources (i.e. arabinose and TCA cycle intermediate) and a nitrogen source (i.e. glutamate) with 0.15 - 0.20 atm of oxygen in the gas phase (Pagan et al. 1975, Kurz and LaRue 1975, LaRue et al. 1976). Nitrogen fixation was also found in static (non-agitated) liquid cultures (Tjepkema and Evans 1975, Evans and Keister 1976), shaking liquid cultures (Keister 1975) and in chemostat cultures (Bergersen and Turner 1976). These findings have been viewed as a major advance in symbiotic nitrogen fixation for several reasons: (1) they have helped to dispel the existing dogma that Rhizobium strains are capable of nitrogen fixation only when associated with leguminous plants, (2) they have conclusively demonstrated that the genetic information for nitrogenase activity is encoded in the bacterium and (3) the derepression of nitrogenase activity in free-living Rhizobium has undoubtedly initiated many investigations aimed towards understanding the regulation of nitrogenase and related assimilatory enzymes. A survey of microsymbionts capable of nitrogenase derepression in asymbiotic cultures is presented in Table 3.1. The most prevalent methods included either the original techniques or modifications thereof on both solid (agar) and liquid media. The agar cultures were derepressed either on agar slants (Kurz and LaRue 1975), on flat agar surface (Pagan et al. 1975, McComb et al. 1975, Rao 1977), on semi-solid agar surface (Finan and Dunican 1980), or using the soft agar overlay method (Pankhurst and Craig 1978) or pellet method (Egeraat et al. 1980).
The liquid media used fell into three categories: liquid stationary cultures (Evans and Keister 1976), rapidly agitating liquid cultures (Keister 1975, Tjepkema and Evans 1975, Keister and Evans 1976, Keister and Ranga Rao 1976), and continuous cultures (Bergersen et al. 1976).

In this chapter, attempts to derepress nitrogenase activity in Rhizobium strains ANU288, ANU289, and NGR231 are described. These strains are capable of nitrogen-fixing symbiosis with the non-legume Parasponia and several tropical legumes such as siratro (Macropihium atropurpureum), stylosanthes and several other plants nodulated by the 'cowpea rhizobia'. Two other strains namely 32H1 and CB756 were used in some comparative experiments. Both of these strains belong to the 'cowpea miscellany' and are capable of nodulating Parasponia. However, these nodules are either ineffective (CB756) or partially effective (32H1) (M. Trinick, pers. comm.). Both these strains are easily derepressed in the free-living vegetative state (Table 3.1) which has led to extensive physiological and biochemical studies (Gibson et al. 1976, Bergersen and Turner 1978, Ludwig 1978, Pankhurst and Craig 1979, Pankhurst 1981, Ching et al. 1981, Kennedy et al. 1981). Since most Rhizobium isolates from Parasponia belong to the 'cowpea miscellany' (Trinick 1980), media combinations similar to those which worked well with other derepressable cowpea strains, were first used to demonstrate the derepression of nitrogenase activity.

Using relatively 'standard' derepression media, it was possible to demonstrate nitrogenase activity in strain ANU289 and its mucoid producing derivative strain ANU288. This chapter describes these studies. It also focuses on the effect on nitrogenase activity of several parameters such as oxygen, pH, carbon dioxide, surface area, growth stage and initial cell density. Additionally various experimental techniques used to derepress nitrogenase in strain ANU289 are described. These were agar slopes,
Table 3.1. A survey of strains examined in different microsymbiont species for in vitro nitrogenase phenotype

<table>
<thead>
<tr>
<th>Microsymbiont</th>
<th>on agar positive</th>
<th>on agar negative</th>
<th>on liquid positive</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC1066, 41F2, 61B9</td>
<td>CC403, NU197</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>127N1, 127NZ, CB1552, CB562, CB627, QA549</td>
<td>32Z3, 29G2, 8B4, 41A1, CB560, CB2236, CB2364, NZP5094, NZP5403, CB744, J641, J2010A, NZP5071, CB1024, 105A1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Parasponia-Rhizobium</strong></th>
<th>ANU288, ANU289</th>
<th>MUI, NGR234,</th>
<th></th>
<th>Mohapatra et al. 1983, McComb et al. 1975</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. lupini</strong></td>
<td>RM11, L5-30</td>
<td>WU274, WU425, CC603, CC624</td>
<td></td>
<td>Pagan et al. 1975, Bedmar and Olivares 1979</td>
</tr>
</tbody>
</table>

Bedmar and Olivares 1979
Table 3.1  (continued)

<table>
<thead>
<tr>
<th>Microsymbiont</th>
<th>on agar positive</th>
<th>negative</th>
<th>on liquid positive</th>
<th>negative</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. meliloti</td>
<td></td>
<td>CC8, SU47, SU388, SU574</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. trifolii</td>
<td>Tlp, T37, T11</td>
<td>TA1, WU95, CC2238C, WA67, 2009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Skotnicki et al. 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lorkiewicz et al. 1978</td>
</tr>
<tr>
<td>R. leguminosarum</td>
<td>TA101</td>
<td>MA505</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycetes:</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mycoplana dimorpha</td>
<td>LLC4, LL-4-9, LL-5-20, LL-8-32, LL-100-32, NCIB 9439</td>
<td>ATCC4279</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. bullata</td>
<td>NCIB9440</td>
<td>ATCC4278</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frankia sp. (Casuarina)</td>
<td>CPII</td>
<td></td>
<td>CPII</td>
<td>D-11, 9-2</td>
<td></td>
</tr>
<tr>
<td>Azospirillum:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. lipoferum</td>
<td></td>
<td>Br17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gauthier et al. 1981</td>
</tr>
</tbody>
</table>
stationary and rapidly agitated liquid cultures. Particular emphasis was given to the strain ANU289, which is highly effective in both Parasponia and a range of legumes. This strain was derived from a P. andersonii nodule isolate CP283 (Trinick 1980).

3.2 Methods

The methods for bacterial culture and growth measurement are described in Section 2.3.1 and the techniques of derepression in Section 2.3.2. Modifications of these techniques if necessary for particular experiments, are described in individual sections of this chapter. The liquid culture techniques for derepression of nitrogenase activity are diagrammatically shown in Fig. 3.1.

3.3 Derepression of nitrogenase activity in agar slopes

The work on nitrogenase derepression on agar slopes was done in collaboration with G. Bender. The agar slope technique is well known for its relative degree of simplicity; there is less stringency for oxygen and nutritional control. However, it should be noted that this technique is only suitable for positive versus negative results. Therefore agar slopes were first used to determine whether the Parasponia - Rhizobium strain ANU289 can be derepressed.

The kinetics of nitrogenase derepression in strain ANU289 grown on agar slopes containing arabinose supplemented with either pyruvate or succinate is shown in Fig. 3.2. Also shown in the same figure are data for strain CB756 previously known to be nitrogenase positive on agar slopes (Pagan et al. 1975, see also Table 3.1). Strain CB756 showed 2 to 4 fold higher rates of acetylene reduction compared to strain ANU289,
Figure 3.1. A diagrammatic presentation of the derepression techniques in liquid cultures. Both stationary and rapid agitation methods are described.
DEREPRESSION TECHNIQUES IN LIQUID CULTURES

- Grow *Rhizobium* to appropriate optical density in RGM30M at 200rpm (28°C)

- Harvest cells, wash and resuspend at 20% original cell density in derepression medium

**STATIONARY METHOD**

- Derepress nitrogenase in 27ml scintillation vial (stoppered) filled with 1.0ml cell suspension under a gas phase of 4% acetylene, 5–10% O₂, and 86–91% argon or helium

- Incubate at 30°C and sample daily (0.2ml) by gas chromatography

- Acetylene reduction protein determination

- O₂/H₂ determination

- Contamination test

**RAPID AGITATION METHOD**

- Incubate 10ml cell suspension in 400ml flat bottomed Wolfe’s bottle under a gas phase of 0.1–0.2% O₂, 4% acetylene and 95.8% helium/argon

- Agitate at 180rpm (30°C)

- Sample every 6 hours for acetylene reduction or oxygen determination
Figure 3.2. Kinetics of nitrogenase derepression in strains ANU289 and CB756 on agar slopes. The induction media was supplemented with either succinate (S) or pyruvate (P) in addition to arabinose as carbon source. Casamino acids (0.05%) served as nitrogen source. The gas phase initially constituted 4% acetylene and the rest air. Values are average of five replicates. The average standard error is shown.
The general pattern of development of nitrogenase activity by strain 289s was similar to strain 756s. A lag period of about 7 days was typical. Both strains inoculated with cyanobacteria supported a significantly higher level of nitrogenase reduction than could be achieved with strain 756s. These studies on slopes confirmed the previous reports (Pankhurst et al. 1982) and showed that the advantage of using 756s to reduce nodule formation in nitrogenase deficient in nitrogenase was similar to strain 289s. The results of these experiments demonstrated that the nitrogenase activity of cyanobacteria is suitable for nitrogenase reduction.
irrespective of carbon sources used. The general pattern of development of nitrogenase activity by strain ANU289 was similar to strain CB756. A lag period of about 7 days was typical. Medium supplemented with succinate supported a significantly higher level of acetylene reduction in both strains compared to medium supplemented with pyruvate. The gas phase above the slope was initially 20% $O_2$ plus 4% acetylene.

These studies on slopes confirmed the previous observation of a requirement for a combination of two carbon sources (e.g. arabinose and succinate, c.f. Pankhurst 1981). Higher activity obtained with succinate was consistent with the results of Kennedy et al. (1981) using a soft agar overlay method (Pankhurst and Craig 1978). Kennedy et al. (1981) used 6-cyanopurine (CPU) in agar medium with the strain CB756 for the selection of nif regulatory mutants. CPU was earlier shown to be a colour indicator for nif expression in K. pneumoniae (MacNeil and Brill 1978). Cen et al. (1982) have used the agar slopes in large scale screening of transposon induced mutants defective in nitrogenase expression in strain CP283, (parent of strain ANU289). This shows that nitrogenase expression on agar slopes, though a relatively simple and inaccurate technique can be used as a valuable tool in Rhizobium nif-genetic analysis.

3.4 Derepression of nitrogenase activity in stationary liquid cultures

Though useful for screening nif positive strains, the agar slope technique suffers from poor resolution with respect to physiological or nutritional studies. The requirements for derepression are known to differ between agar slopes and liquid culture techniques. Having been able to derepress strain ANU289 in agar slopes, the derepression of nitrogenase was attempted in stationary liquid cultures using 27ml scintillation vials.
3.4.1 Time course of derepression

Figure 3.3 shows the kinetics of nitrogenase activity in Parasponia-Rhizobium strains ANU288, ANU289, and NGR231 and 32H1. With the exception of NGR231, nitrogenase activity was detected within 4 to 7 days and then went through an exponential phase followed by cessation of fixation after 16 days. Strain ANU289 showed higher activity than the control strain 32H1 under the tested conditions. Under similar conditions, strain ANU288 was derepressed and maximally produced about 80% of the activity of strain ANU289.

Table 3.1 shows the enormous variation within a Rhizobium species with regard to the ability to develop nitrogenase activity. Several strains do not express activity despite extensive efforts involving variations in both media and techniques. For example, Rhizobium strain MUL isolated from Parasponia rugosa (previously called Trema cannabina) was shown to be nitrogenase negative on agar slopes (McComb et al. 1975). NGR231, an isolate from P. rugosa nodules (Trinick 1973) did not develop nitrogenase activity. In contrast to strains ANU288, ANU289, CB756 and 32H1, strain NGR231 is not rifampicin resistant. This supports the correlation observed by Pankhurst et al. (1982) between rifampicin (an inhibitor of bacterial RNA polymerase, Wehrli and Staehelin 1971) resistance and the capacity for in vitro nitrogenase derepression in rhizobia. However, NGR231 mutant strains isolated for rifampicin resistance failed to show in vitro nitrogenase activity (data not shown).

Strain ANU289 in stationary culture showed a lag phase of only 3-4 days in contrast to 7-10 days for agar slopes. Furthermore, the specific rates were higher in liquid cultures. However, the oxygen concentration in the gas phase was reduced from atmospheric levels used in agar slopes to 10% initial O₂ concentration in the stationary technique. The low activity in strain 32H1 may be attributed to the lower O₂ level in the gas phase and a less favourable nutritional environment (e.g. casamino
Figure 3.3. Kinetics of nitrogenase derepression in *Parasponia*-Rhizobium strains ANU288 and ANU289 and NGR231 as well as cowpea strain 32H1 in stationary liquid cultures. The derepression media were supplemented with 50mM arabinose and 20mM succinate (as carbon sources) and 0.05% casaminoacids (as nitrogen source). Cells were incubated in 27 ml vials with a gas phase initially containing 5% oxygen. Nitrogenase activity was measured as nmoles of ethylene produced per vial. Values are average of five replicates. The error bar represents the average of standard errors.
Effects of oxygen concentration in the gas phase

The results obtained have shown that there was a significant difference in the gas phase and that the percentage of nitrogen activity in the gas phase is very low. This is in contrast to the results of earlier studies, which showed that the gas phase is a very narrow range compared to the oxygen concentration. The study of ANO288 in the gas phase in a very narrow range is important for understanding the nitrogen activity in the gas phase. Therefore, the stationary culture technique is not suitable for determining the precise oxygen requirements. For this reason, oxygen requirement studies in rapidly growing cultures are more relevant (see 3.4.3 of this chapter and Chapter 7.2).
acids 0.05%) in the derepression medium. Optimal activity was reported for strain 32H1 with 0.1% casamino acids (as nitrogen source) and 0.1% gluconate (as carbon source) and 20% O₂ in the gas phase (Evans and Keister 1976).

3.4.2 Effect of oxygen concentration in the gas phase

The effect of different levels of oxygen in the gas phase was tested (Fig. 3.4) on derepression kinetics of nitrogenase activity in strain ANU289. Oxygen levels of 0, 5, 10, 20, 30 and 40% in the gas phase were tested. The 5 and 10% oxygen treatment resulted in maximal derepression in strain ANU289. Concentrations of oxygen lower than 5% or higher than 10% gave significantly lower amounts of acetylene reduction. No acetylene reduction was seen in the absence of oxygen indicating that oxygen was required.

Thus, the results obtained here clearly showed that the oxygen concentration in the gas phase was of paramount importance in the development of nitrogenase activity in liquid cultures. The window of 5 to 10% in the gas phase is a very narrow optimum range compared to other 'cowpea strains' such as 32H1 and CB756 (see Chapter 7.3). Evans and Keister (1976) reported maximal activity with 10 and 20% oxygen in the gas phase in strain 32H1 using a similar experimental approach. An oxygen level of 20% (v/v) oxygen in the gas phase was not conducive for nitrogenase derepression in strain ANU289. It appears likely that rhizobia at the bottom of culture vials are mainly involved in acetylene reduction and that there may be an oxygen gradient through the liquid phase. Therefore, the stationary culture technique is not suitable for determining the precise oxygen requirement. For this reason, oxygen requirement studies in rapidly agitated cultures are more relevant (see 3.4.1 this chapter and Chapter 7.2). The oxygen requirement in Parasponia - Rhizobium strains are of particular significance in relation to a
Figure 3.4. Effect of oxygen on derepression of nitrogenase activity in strain ANU289. The medium J20 Arab contained 50mM arabinose and 20mM succinate (as carbon sources) and 0.05% casamino acids as nitrogen source. Cells were incubated in 27 ml vials with a gas phase initially containing different levels of oxygen as shown in the figure. Nitrogenase activity was measured as nmoles ethylene per mg protein. Values are average of 10 replicates. The error bar represents average standard error.
3.4.1 Effects of washing of immortal cells

Cells are normally pre-cultured in a growth medium before (day 0) and transferred to a dempression medium. Since the growth medium and dempression medium are never the same, it is expected that the procedure would increase the ethylene level.

A graph presents data on the effect of washing. The cells were grown on a 20% (1.5 x 10⁶ cells/mL) in DM with washing 0.05M phosphate buffer with dempression medium at 37°C. Washed cultures were then transferred and resuspended in the dempression medium. On the graph, the percentage of the activity appeared simultaneously in both control and washed cells, with activity appearing days; however, the washed cells rapidly fell though the control cells.

The reduction between washed and unwashed cells is significant. Development of cell density is dealt with in detail in Section 3.4.4. In this experiment, cell washed in DM containing 1% glutamine and 1% metyrapone was washed with 0.05M phosphate buffer with dempression medium. There was no difference between unwashed and washed cells.

3.4.4 Effect of cell density and growth stage

One of the critical factors controlling methane reduction in liquid culture was considered to be the initial cell concentration. It was empirically observed in the previous experiment that altering cells three-fold gave maximal ethylene reduction. Additionally, the duration of cell culture during the pre-growth period was dependent on the growth
report of the absence of a legume-type leghemoglobin in *Parasponia* (Coventry et al. 1976) nodules. This will discussed in detail in Chapter 7 and Chapter 8.

### 3.4.3 Effects of washing of inoculant cells

Cells are normally pre-cultured in a growth medium before they are transferred to a derepression medium. Since the growth medium and derepression medium are never the same, it is expected that the preculture would influence derepression of nitrogenase activity.

Table 3.2 presents data on the effect of washing the cells in derepression medium before incubation. Cells pre-grown to a density of 280 Klett units (2-2.5 x 10^9 cells/ml) in BMM were either diluted by 1 to 3 fold with derepression medium or spun, washed in nitrogen free B5 medium and resuspended in the derepression medium in the same proportion. The activity appeared simultaneously in both unwashed and washed cells after 3 days, however, the washed cells rapidly went through the exponential phase. No difference was found in the total amount of acetylene reduction between unwashed and washed cells after 17 days. The effect of cell density is dealt with in detail in Section 3.4.4.

In this experiment, cells were grown in BMM containing 1% mannitol (carbon source), 0.05% glutamate and 0.05% yeast extract (as nitrogen source) before being used for a derepression experiment. The longer lag period in unwashed cells in contrast to washed cells may be due to carry over from the pre-culture medium of substances inhibitory to nitrogenase derepression. This conclusion is supported by the fact that with cells pre-grown in the derepression medium, there was no difference between unwashed and washed cells.

### 3.4.4 Effect of cell density and growth stage

One of the critical factors controlling acetylene reduction in liquid cultures was considered to be the initial cell concentration. It was empirically observed in the previous experiment that diluting cells three-fold gave maximal acetylene reduction. Additionally, the increase in cell number during the pre-growth period was dependent on the growth
Table 3.2. Comparison of acetylene reduction in *Rhizobium* strain ANU289 with diluted (unwashed) and spun (washed) cells

<table>
<thead>
<tr>
<th>cell density</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
<th>10 days</th>
<th>17 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diluted:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>1.2</td>
<td>3.6</td>
<td>3.7</td>
<td>3.8</td>
<td>10.9</td>
</tr>
<tr>
<td>1:2</td>
<td>2.9</td>
<td>25.0</td>
<td>41.8</td>
<td>210.1</td>
<td>274.5</td>
</tr>
<tr>
<td>1:3</td>
<td>5.3</td>
<td>38.1</td>
<td>71.1</td>
<td>292.4</td>
<td>293.4</td>
</tr>
<tr>
<td>Control</td>
<td>0.6</td>
<td>2.0</td>
<td>2.5</td>
<td>6.7</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Spun:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>11.3</td>
<td>21.0</td>
<td>24.0</td>
<td>25.3</td>
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<td>72.0</td>
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<td>6.7</td>
<td>119.8</td>
<td>220.8</td>
<td>368.3</td>
<td>370.6</td>
</tr>
<tr>
<td>Control</td>
<td>12.6</td>
<td>15.0</td>
<td>15.2</td>
<td>16.3</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Arabinose (50mM) and succinate (20mM) were used as carbon sources and casamino acids 0.05% served as nitrogen source. Oxygen was 5% (v/v) in gas phase. Control:1 ml of cells from preculture medium (BMM) directly transferred to derepression vial. Otherwise cells from preculture medium were directly diluted or washed and resuspended in derepression medium at 1:1, 1:2 and 1:3. Data represent acetylene reduction in nmoles of ethylene per vial 27 ml over days and are average of five replicates.
Figure 3.5. Effect of (A) cell density and (B) growth stage on derepression of nitrogenase activity in strain ANU289. The medium J20 Arab contained 50mM arabinose and 20mM succinate (as carbon sources) and 0.05% casamino acids (as nitrogen source). (A) cells were harvested at Klett 240 were washed and resuspended in an equal volume of J20 Arab medium. These cells were then differentially diluted by the addition of various volumes of same medium (e.g. 1:4 means 4 parts of J20 Arab was added to the washed cells giving rise to cell density of 20%). (B) cells harvested at different periods of growth were washed and diluted at 1:4. Experiments were done with 1 ml cells in 27 ml vials with a 5% C2 in gas phase. Nitrogenase activity was measured as nmoles of ethylene per vial. Values are average of five replicates.
DAYS

300

200

100

nmols ethylene/  

DAYS

300

200

100

0

0

2

4

6

8

DAYS

1:4

1:3

1:2

1:1

nmols ethylene/vial

35 6 8
medium. Cell number at harvest was also growth stage dependent (see Section 2.3.1). Therefore, the effect of initial cell concentration on development of nitrogenase activity in strain ANU289 was investigated. The data are presented in Fig. 3.5A. Cells grown to mid-logarithmic stage (Klett 240) in BMM, washed by centrifugation and resuspended in the derepression medium to a density of about 20% of the original cell concentration gave the highest activity. Increased cell density reduced the total activity. Final protein concentrations per vial of differentially inoculated trials were similar, indicating that the higher dilutions with lower initial cell number grew more, while the undiluted cells remained nearly stationary. The growth stage directly influenced development of nitrogenase activity, probably via cell number. As is shown in Fig. 3.5B, cells harvested at mid- to late-logarithmic stage (Klett unit 240-300) in BMM gave best results, in terms of total nitrogenase activity and a shorter lag period. If the stationary phase cells were used at a cell density of 5 to 10% (in contrast to the usual mid-log phase cells at 20% cell density), optimal activity was obtained after 15 days (as compared to optimally 7 to 10 days).

These findings, thus, demonstrate the important modifying effects on derepression of initial cell density and growth stage. In fact, the interplay of these two parameters was found to produce some variability between separate experiments.

3.4.5 Effect of pH and carbon dioxide

Several years ago, Lowe and Evans (1962) demonstrated that several species of rhizobia required CO₂ for aerobic growth. CO₂ was reported to stimulate acetylene reduction markedly in the R. japonicum strain 311b83 (Keister and Ranga Rao 1976). In the same study they also observed that a large shift in pH was caused by dissolved CO₂ in the medium. Experiments with strain 32H1 showed that a higher concentration of
Table 3.3. Effect of pH and carbon dioxide on acetylene reduction (AR) in strain ANU289

<table>
<thead>
<tr>
<th>Buffers (pH)</th>
<th>no CO₂</th>
<th>1% CO₂</th>
<th>2.5% CO₂</th>
<th>5% CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES (6.03)</td>
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<td>6.0</td>
<td>152</td>
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<tr>
<td>HEPES (6.49)</td>
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<td>6.4</td>
</tr>
<tr>
<td>MOPS (7.0)</td>
<td>85</td>
<td>6.9</td>
<td>105</td>
<td>6.7</td>
</tr>
<tr>
<td>TRICINE (8.07)</td>
<td>0</td>
<td>7.9</td>
<td>0</td>
<td>8.0</td>
</tr>
<tr>
<td>Control (6.5)</td>
<td>0</td>
<td>8.2</td>
<td>12</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Arabinose 50mM and succinate 20mM (carbon sources), casamino acids 0.05% (nitrogen source) were used. The oxygen levels in the gas phase was 5% (v/v). Nitrogenase activity is expressed as nmoles of ethylene per mg protein. Values are average of five replicates. Abbreviations: MES = (2-[N-Morpholino]-ethane sulfonic acid), HEPES = [4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid], MOPS = [3-(N-morpholino) propane sulphonic acid], TRICINE = [N-(tris-(hydroxy methyl)-methyl) glycine]. Unbuffered derepression medium served as control.
phosphate was somewhat stimulatory, presumably through a pH buffering effect (Gibson et al. 1976, Evans and Keister 1976).

The medium used in this chapter did not use a high phosphate concentration. This warranted an examination of the effect of pH. As shown in Table 3.3, pH clearly affected acetylene reduction in strain ANU289. No marked stimulation was observed by CO₂ in this strain, even though a large reductions in the pH of the medium were caused by the addition of 2.5 and 5% carbon dioxide in the gas phase. No shift in pH occurred in the control (no carbon dioxide) and vials injected with 1% carbon dioxide. In contrast to Keister's work, the pH rose rapidly in the medium perhaps due to succinate metabolism. Detectable nitrogenase was negligible, when the pH exceeded 7.5. Optimal activity was obtained at pH 6.5. Thus, although 2.5 - 5% CO₂ had a buffering capacity the CO₂ had no effect on nitrogenase derepression under these conditions.

Thus, in strain ANU289 pH has a definite effect on acetylene reduction similar to that found in several other studies with strain 32H1 (Keister 1975, Gibson et al. 1976, Pankhurst 1981). However, unlike 32H1, no significant stimulation in activity was obtained in this strain by adding 1% CO₂.

3.4.6 Effect of surface area and gas phase on specific activity

All previous experiments were done using 27ml scintillation vials with 1 to 1.5ml total volume of liquid medium. Increasing this volume to more than 1.5ml reduced the total activity. This suggested a possible effect of the relative surface area or liquid depth (and thus oxygen gradients) on fixation. Keeping the depth of the liquid phase constant, a significant increase in specific activity was obtained, when the surface area was increased.

Data in Table 3.4 demonstrate the correlation between specific activity and surface area by using Corning tissue culture flasks. The increase was not proportional, as an increase of surface area from 4.5 to
Table 3.4. Effect of increasing surface area on specific activity in strain ANU289

<table>
<thead>
<tr>
<th>Flasks</th>
<th>Total Capacity (cc)</th>
<th>Surface Area (cm²)</th>
<th>Gas Phase Ratio</th>
<th>Specific Activity (nmoles/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scintillation vial</td>
<td>27</td>
<td>4.5</td>
<td>6.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Small Corning tissue culture flask</td>
<td>70</td>
<td>48</td>
<td>1.46</td>
<td>18.6</td>
</tr>
<tr>
<td>Large Corning tissue culture flask</td>
<td>240</td>
<td>75</td>
<td>3.43</td>
<td>50.2</td>
</tr>
</tbody>
</table>

Mannitol (50mM) and succinate (20mM) were used as carbon sources. Sodium glutamate (3mM) served as nitrogen source. The flasks were gased with a mixture of 5% O₂, 4% C₂H₂ and 91% Helium. Specific activity was calculated between day 6 and day 8. The values are average of five replicates.
75cm², increased specific activity about 10 fold. The Corning flask did not show a proportional increase suggesting that the surface area to gas phase ratio may be more important than the surface area itself.

Undoubtedly in these early experiments the specific activity in strain ANU289 was much lower as compared to 32H1 and CB756. Results from Table 3.4 show that by altering the surface area to gas phase ratio, a significant increase in activity could be obtained. The importance of this ratio was emphasized by Egeraat et al. (1980), who claimed that acetylene reduction by fast growing strains was found when the ratio of agar-surface to air volume was high. The effect of surface area to gas phase ratio may reflect any oxygen mediated effect, i.e. microaerobic conditions may be needed for some cell proliferation, which then depletes nutrients (and possibly nitrogenous compounds), so that subsequent sub-microaerobic conditions permit nitrogenase activity. These results need to be viewed with caution as no further published work has appeared and one might be looking at slow growing contaminants. Be this as it may, the general conclusion, however is the same.

Thus, by using flat-bottomed Corning 75cm² tissue culture flasks larger quantities of derepressed cells were obtainable (equivalent to 30 scintillation vials), but the specific activity was increased 10 fold. This has significance when large amounts of derepressed cells are required for biochemical or molecular studies, such as in RNA isolation or enzymological analysis of "fixing" cultures.

3.5 Derepression of nitrogenase in rapidly agitating cultures

Nitrogenase derepression in rapidly agitating cultures required a relatively more complex experimental design. It required finer control of oxygen concentrations in the gas phase but it permitted better control over nutritional and physiological parameters. The requirements for
nitrogenase derepression differed from those of agar slopes or stationary liquid cultures and these are discussed below.

3.5.1 Oxygen requirement in rapidly agitating cultures

The effect of various concentrations of oxygen on the initiation and rate of derepression of nitrogenase activity for Rhizobium strain ANU289 are shown in Fig 3.6. The concentrations of oxygen shown were the average of oxygen readings taken at 8 hour intervals. The range of oxygen readings is given in the figure legend. A moderate oxygen concentration ratio of 0.25% (V/V) was optimal for rapid initiation of activity. Higher and lower oxygen concentrations affected derepression either by delaying the onset of activity or lowering specific rates. No acetylene reduction was found with no oxygen in the gas phase and with oxygen concentrations at higher than 0.5% in the gas phase. The lag period was dependent upon the level of oxygen used and varied from 24 to 48 hours.

Thus the oxygen requirement of strain ANU289 in rapidly agitating culture was fairly low, similar to strain 32H1 and 311b83 (Keister and Evans 1976), and 61A76 (Keister and Ranga Rao 1976).

3.5.2 Influence of cell density

The other critical parameter governing derepression kinetics was the cell density. Table 3.5 describes the effect of different cell densities on derepression of nitrogenase activity in strain ANU289. An initial cell density of $1 \times 10^9$ cells/ml showed the highest specific rates and maximal total activity. Slightly higher ($1.65 \times 10^9$ cells/ml) or slightly lower ($5.5 \times 10^8$ cells/ml) initial cell densities resulted in a decline in activity. The final protein was similar irrespective of differential inoculum. The effect of cell density was previously observed in strain 32H1 (Keister and Evans 1976), where the highest specific rates were obtained when the optical density of the inoculant cells was between 0.04 - 0.06 (at 680nm) but with lower activities when densities below or
Figure 3.6 The effect of oxygen on the acetylene reduction with Parasponia-Rhizobium strain ANU289. Na-succinate (50 mM) and Na-glutamate (3 mM) served as carbon and nitrogen sources, respectively. The medium was buffered with 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid pH 6.8-7). (A) Time course of derepression at different oxygen levels. The oxygen concentrations given are the average of readings taken at approximately 8 hour intervals. The ranges were 0.11% (0.08-0.15), 0.21% (0.17-0.25), 0.25% (0.24-0.26), 0.35% (0.33-0.40), 0.47% (0.43-0.50%). The ordinate is total amount (in nmoles) of ethylene in 400 ml flask containing 10 ml of culture.
Table 3.5. Effect of cell density on nitrogenase derepression with strain ANU289

<table>
<thead>
<tr>
<th>Initial cell density (viable cells/ml)</th>
<th>Final protein mg/ml</th>
<th>Specific activity nmoles C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.050</td>
<td>42 ± 14</td>
</tr>
<tr>
<td>1.1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.075</td>
<td>68 ± 9</td>
</tr>
<tr>
<td>1.65 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.050</td>
<td>26 ± 14</td>
</tr>
</tbody>
</table>

Succinate (50mM) and glutamate (3mM) served as carbon and nitrogen source, respectively. The medium was buffered with HEPES (pH 6.8). Oxygen concentrations were adjusted to 0.2-0.25% O<sub>2</sub> (v/v) in the gas phase every 12 h. Specific activity was determined during 60-72 hours. Values are average of five replicates with ± SE.
above this were used. Findings on ANU289 suggested that these physiological requirements probably hold true for many *Rhizobium* strains. Thus oxygen concentration and initial cell density appeared to be two important parameters in the development of nitrogenase activity. With increasing oxygen concentration a prolonged lag was noticed. Only oxygen concentrations of 0.25% in the gas phase coupled with an initial cell density of $1.1 \times 10^9$ ml gave the maximum specific rates. This was presumably due to the fact that at very low cell densities, excess oxygen inhibited nitrogenase, whereas at high cell densities, the oxygen was rapidly consumed.

### 3.6 Summary

Experiments in this chapter clearly establish that of the three *Parasponia*-Rhizobium strains tested (ANU288, ANU289 and NGR231), it is possible to derepress nitrogenase activity in strain ANU289. Activity can be detected in agar slopes, stationary liquid cultures and rapidly agitating cultures. Each of these techniques has its advantages and disadvantages, and each has its application. For instance, agar slopes can be used in large scale screening of Tn5 induced mutants for nitrogen fixation defects. Similarly stationary liquid culture provides a simpler means (because continuous adjusting of oxygen levels is not necessary) of producing sufficient derepressed cells for nif mRNA isolation and thus can be of help in molecular genetic studies.

In stationary liquid culture experiments the specific activity 4-5 n moles/hr/mg protein is much lower than the specific activity previously reported for strains 32H1 (Keister and Evans 1976, Keister and Ranga Rao 1976, Pankhurst 1981) and CB756 (Bergersen et al. 1976). But it is also clear from these experiments that there is a large scope for increase in activity. For example simply by changing over from scintillation vials
scintillation vials to tissue culture flasks, specific activity was increased by 10 fold in stationary cultures. Further optimisation of the nutritional environment may further increase the activity.

The more sophisticated rapidly agitating culture method permits a detailed study of physiological parameters particularly regarding oxygen requirements and sensitivity. In rapidly agitating cultures, the results illustrate that the critical factors for derepression of nitrogenase activity are the oxygen concentration and the cell density.
CHAPTER-4

NUTRITIONAL CONTROL

OF

IN VITRO NITROGENASE ACTIVITY
4. NUTRITIONAL CONTROL OF IN VITRO NITROGENASE ACTIVITY

4.1 Background

The positive reports of nitrogenase derepression are restricted to a few strains of the slow growing *R. japonicum* and *Rhizobium* sp. (cowpea rhizobia, Table 3.1). Several strains belonging to these species cannot be derepressed for nitrogenase under similar conditions to those used for other positive strains. Keister and Evans (1976) found that the major determinant in the expression of nitrogenase activity in the strain which they examined, was a consistently low oxygen tension during the experiment.

Though the mechanism has not been clearly elucidated, some other reports (Pankhurst 1981, Agarwal and Keister 1982) indicated that physiological parameters other than a low oxygen tension may be involved in nitrogenase synthesis and activity in free-living cultures of *Rhizobium*. Pankhurst (1981) examined three different *Rhizobium* strains and confirmed that the provision of appropriate nutritional conditions was a necessary pre-requisite for the synthesis of nitrogenase by agar cultures of *Rhizobium*. It was also shown that *Rhizobium* strains differ with regard to nutritional requirements for the expression of this enzyme. Agarwal and Keister (1982) used 36 *R. japonicum* strains for derepression studies in liquid cultures and reached the same conclusion.

Thus for the strains reported as nitrogenase negative, it may be that as yet, optimal conditions for derepression in these strains have not been found. *R. japonicum* strain 3I-1b-138 is a good example, since on defined media it was first classified nitrogenase negative (Reporter and Hermina 1975), then as occasionally derepressable (Agarwal and Keister 1982) and finally as nitrogenase positive (Gresshoff 1982).
In the absence of any concrete evidence in favour of the genetical nature of strain-difference in terms of in vitro nitrogenase derepression, and from the reports of the alternative nutritional requirements for different Rhizobium strains (Pankhurst 1981, Agarwal and Keister 1982) for optimal activity, it appears that nutritional parameters play an important role in derepression of nitrogenase in any Rhizobium strain.

In the preliminary experiments of derepression of nitrogenase activity in Rhizobium strain ANU289 outlined in the previous chapter, the nutritional conditions used for other 'cowpea rhizobia' were employed. In most experiments, the medium used contained arabinose 50mM, succinate 20mM (as carbon sources) and casamino acid 0.05% (as nitrogen source), iron and molybdenum (as in tissue culture medium such as B5, Gamborg and Eveleigh, 1968). However, in view of the preceding, the standard 'arabinose-succinate-casamino acid' medium may not be optimal for Rhizobium strain ANU289. Therefore, the nutritional requirements in terms of carbon sources, nitrogen sources, iron, molybdenum and cyclic nucleotides of this strain have been studied further and are presented in this chapter.

Agar slopes are unsuitable for this study because of the lack of fine control on nutritional composition and utilization, variable growth of inoculant, formation of extreme oxygen gradients, low specific activities and longer (11-15 days) lag periods compared to those of 3-7 days seen in stationary culture. Shaken liquid cultures (see Chapter 3) are also impracticable for most of such studies due to the difficulty in handling a large number of experimental replicates and the need for continuous monitoring of the gas phase to maintain the 'correct' oxygen levels. Therefore, the experiments reported in this chapter were done in stationary liquid culture by maintaining appropriate oxygen concentrations
in the gas phase, some major conclusions have been verified by the rapidly agitated culture method.

4.2 Methods

The techniques of nitrogenase derepression have been described (Section 2.3.2). For experiments described in this chapter, the stationary liquid culture technique was mostly used. Carbon and nitrogen sources to be tested were separately prepared, filter sterilized and added to the basic media at the required concentration. Similarly, the effect of molybdenum was tested by preparing a trace element stock (Gresshoff and Doy 1974) devoid of molybdenum and then adding molybdenum at the desired concentration. Various sources of iron such as iron chelate, iron citrate or ferric chloride were separately prepared at varying concentrations and added to the basic media before autoclaving. The effects of cyclic adenine nucleotides were, however, tested in a different manner. Cells were first derepressed using 'standard' derepression media. Freshly prepared and filter-sterilized stocks of various cyclic nucleotides were then injected through the suba seal. Results obtained in stationary liquid cultures were sometimes repeated in rapidly agitating liquid cultures (Section 2.3.2), not shown in this chapter.

4.3 Requirements of carbon sources for nitrogenase expression

In several slow growing rhizobia grown on solid media, Pagan et al. (1975) found arabinose or galactose together with either succinate or fumarate suitable for nitrogenase activity. Kurz and LaRue (1975) and LaRue et al. (1976) reported that one carbon source (xylose or arabinose) sufficed for some strains, but that two carbon sources were necessary for others. Reasonable activity was also reported with succinate alone as a carbon source in Rhizobium sp. 32H1 (McComb et al. 1975). In contrast,
Gibson et al. (1976) reported a need for three carbon sources, i.e., inositol and succinate with either ribose or arabinose for optimal activity. A combination of two carbon sources (a pentose sugar, e.g., arabinose and a TCA cycle intermediate, e.g., succinate) was advocated by Pankhurst (1981).

High specific activities of nitrogenase were obtained with gluconate in stationary liquid cultures of strain 32H1 (Evans and Keister 1976). In liquid shake cultures, gluconate or succinate was the preferred carbon source for *R. japonicum* strain 61A76, USDA83 and strain 32H1 (Keister and Evans 1976). Malate also promoted *in vitro* nitrogenase activity in *Rhizobium* sp. 32H1 (Tjepkema and Evans 1975) using a similar culture system. In continuous cultures used by Bergersen et al. (1976), a combination of either glycerol and succinate or arabinose and succinate was found most suitable for nitrogenase synthesis in *Rhizobium* sp. 32H1 and CB756.

The experiments described in Chapter 3 with strain ANU289 utilized D-arabinose (50mM) and succinate (20mM) for derepression. The effect of replacing D-arabinose by twelve other sugars and succinate by eleven other organic acids on growth and nitrogenase activity is described in this section.

### 4.3.1 Growth on various sugars

The relative aerobic growth of strain ANU289 on different sugars (Table 4.1) in shaken liquid cultures was tested. Strain ANU289 was able to utilize primarily all the pentoses and fairly well all the hexose sugars. Exceptionally vigorous growth was seen in the case of fructose. Disaccharides and sugar alcohols supported very poor growth.
Table 4.1. Growth of Parasponia-Rhizobium strain ANU289 on different sugars and organic acids in liquid aerobic culture

<table>
<thead>
<tr>
<th>substrate</th>
<th>relative growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>sugars</td>
<td></td>
</tr>
<tr>
<td>Pentoses:</td>
<td></td>
</tr>
<tr>
<td>D-arabinose</td>
<td>+++</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+++</td>
</tr>
<tr>
<td>D-xylose</td>
<td>+++</td>
</tr>
<tr>
<td>D-ribose</td>
<td>+++</td>
</tr>
<tr>
<td>Hexoses:</td>
<td></td>
</tr>
<tr>
<td>D-mannose</td>
<td>++</td>
</tr>
<tr>
<td>D-glucose</td>
<td>++</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+++</td>
</tr>
<tr>
<td>D-galactose</td>
<td>++</td>
</tr>
<tr>
<td>Disaccharides:</td>
<td></td>
</tr>
<tr>
<td>D-sucrose</td>
<td>-</td>
</tr>
<tr>
<td>D-maltose</td>
<td>+</td>
</tr>
<tr>
<td>sugar alcohols:</td>
<td></td>
</tr>
<tr>
<td>D-mannitol</td>
<td>+</td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>D-inositol</td>
<td>-</td>
</tr>
<tr>
<td>organic acids</td>
<td></td>
</tr>
<tr>
<td>acetate</td>
<td>++</td>
</tr>
<tr>
<td>citrate</td>
<td>+++</td>
</tr>
<tr>
<td>fumarate</td>
<td>+</td>
</tr>
<tr>
<td>gluconate</td>
<td>+++</td>
</tr>
<tr>
<td>glyoxylate</td>
<td>++</td>
</tr>
<tr>
<td>isocitrate</td>
<td>++</td>
</tr>
<tr>
<td>ketoglutarate</td>
<td>++</td>
</tr>
<tr>
<td>malate</td>
<td>+++</td>
</tr>
<tr>
<td>malonate</td>
<td>+</td>
</tr>
<tr>
<td>pyruvate</td>
<td>++</td>
</tr>
<tr>
<td>succinate</td>
<td>++</td>
</tr>
</tbody>
</table>

Growth was monitored at stationery phase which was attained after 5-10 days of culture.

- = no growth (no increase)
+ = trace to poor growth (OD650 0.2)
++ = fair growth (OD650 0.2-0.4)
+++ = good growth (OD650 0.4-0.7)
++++ = vigorous growth (OD650 >0.7)

Sugars and sugar alcohols were tested at 50mM; organic acids at 20mM; ammonium chloride (10mM) was present as a nitrogen source.
Similar to other slow growers, strain ANU289 was unable to utilize disaccharides such as sucrose and could only poorly utilize maltose or lactose. Robertson and Taylor (1973) reported the presence of an invertase in _R. lupini_ bacteroids. Singh et al. (1980) reported an invertase in a _R. japonicum_ strain isolated from _Sesbania grandiflora_. This enzyme hydrolysed sucrose extra-cellularly. Later, however, slow growing cultured cells of _Rhizobium_ sp. (NGR46, 32H1), _R. lupini_ (WU8, WU425) and _R. japonicum_ (CB1809) were shown to lack an active uptake system for $^{14}$C labelled disaccharides (Glenn and Dilworth 1981). Additionally, _R. lupini_ bacteroids were unable to use $^{14}$C lactose or glucose as were fast growing _R. leguminosarum_ bacteroids. _R. japonicum_ bacteroids from soybean nodules, were however shown to have both maltase and trehalase. Growth studies (Table 4.1) with ANU289 indicated that maltose and lactose were used poorly, but no growth whatsoever was found with sucrose. This was perhaps due to lack of invertase, as in other slow growing rhizobia.

4.3.2 Utilization of TCA cycle intermediates and related compounds

Among organic acids tested, strain ANU289 was capable of growth on all organic acids with varying degrees (Table 4.1). The growth was poor for malonate and fumarate or moderately good as was the case of acetate, glyoxylate, succinate, isocitrate, α-keto-glutarate and pyruvate. The growth on malate and gluconate was exceptionally good and very similar to that seen on fructose.

Graham (1964) showed that many strains of seven species of _Rhizobium_ (belonging to both the fast and slow growing types) would
utilize pyruvate and the intermediates fumarate, malate and succinate and citrate in a *R. japonicum*, *R. lupini* and Rhizobium sp. From their growth pattern on various TCA cycle intermediates and related compounds Skotnicki and Rolfe (1978) proposed an incomplete TCA cycle in cowpea rhizobia. Results shown in Table 4.1 are not in agreement with general growth-patterns of those strains (Skotnicki and Rolfe 1978), since strain ANU289 was more or less capable of growth in all compounds tested. However, lack of, or poor growth obtained at the 20mM concentration in the work by Skotnicki (1978) may be because of plate growth conditions or more importantly lack of pH control (alkalinity shifts may occur due to uptake). In contrast the results for strain ANU289 were obtained in adequately buffered liquid cultures.

4.3.3 Effect of sugars on nitrogenase derepression

Various sugars were tested as alternatives for arabinose and scored for their ability to support nitrogenase activity (Table 4.2). The derepression medium thus contained the basic nutrients, 0.05% casamino acids as nitrogen source, succinate (20mM) and the tested sugar source at 50mM. Very little activity was observed in media with no added carbon source. Succinate supplied alone (as a control), however, gave higher activity than succinate plus D-arabinose. Interestingly, L-arabinose showed higher activity than D-arabinose, although it was shown (Table 4.2) that both can serve as efficient carbon sources for growth of this strain. Strain ANU289 developed higher nitrogenase activity in media supplemented with hexose sugars and disaccharides. For example, if D-arabinose was replaced with D-mannitol a six-fold higher activity was obtained.
Mannitol plus succinate showed threefold higher nitrogenase activity than with succinate alone. With the sugars which supported better activity, the lag period before detection of activity was longer than with D-arabinose, but the total duration of activity was extended. No correlation was found between nitrogenase activity and growth in terms of final protein concentration, although the cultures with higher activity were generally less mucoid.

Activity observed in media supplemented with both succinate and D-arabinose were in agreement with previous findings (Chapter 3). Higher activity observed with L-arabinose than D-arabinose was also observed by Wilcockson and Werner (1979) in R. japonicum strain 61A76. In contrast to other cowpea rhizobia, higher nitrogenase activity was obtained with media supplemented with hexose sugars, disaccharides or sugar alcohols. The requirement for a combination of a sugar and a TCA cycle intermediate in stationary culture was apparent from Table 4.2 where a combination such as mannitol plus succinate stimulated three-fold the expression of nitrogenase in strain ANU289 compared to succinate alone. Furthermore, higher activity with succinate alone than arabinose alone and arabinose plus succinate indicate that succinate may play a major role in the cellular metabolism during nitrogen fixing conditions. It may interact with the sugar present either synergistically or additively to effect or affect the nitrogenase expression. For example, since rhizobia produce acid from sugar and alkali from organic acids, supplementation with both may help to maintain pH (Ronson et al. 1981). Alternatively, succinate respiration or uptake may cause the cell to use the non-utilizable sugars or perhaps restrict the utilization of sugars otherwise rapidly metabolized.
Table 4.2. Nitrogenase activity of strain ANU289 on basic media supplemented with different sugars

<table>
<thead>
<tr>
<th>sugar compounds tested</th>
<th>activity after ²</th>
<th>growth after 30d ²²³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8d</td>
<td>16d</td>
</tr>
<tr>
<td>no carbon</td>
<td>0</td>
<td>17.2</td>
</tr>
<tr>
<td>succinate (suc)</td>
<td>51.1</td>
<td>158.5</td>
</tr>
<tr>
<td>Pentoses:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc + D-arabinose</td>
<td>156.8</td>
<td>250.0</td>
</tr>
<tr>
<td>Suc + L-arabinose</td>
<td>130.7</td>
<td>286.4</td>
</tr>
<tr>
<td>Suc + D-xylose</td>
<td>63.8</td>
<td>135.7</td>
</tr>
<tr>
<td>Hexoses:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc + D-mannose</td>
<td>153.3</td>
<td>509.7</td>
</tr>
<tr>
<td>Suc + D-glucose</td>
<td>67.6</td>
<td>243.2</td>
</tr>
<tr>
<td>Suc + D-fructose</td>
<td>159.9</td>
<td>279.3</td>
</tr>
<tr>
<td>Suc + D-galactose</td>
<td>47.8</td>
<td>144.7</td>
</tr>
<tr>
<td>Disaccharides:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc + D-sucrose</td>
<td>141.3</td>
<td>374.4</td>
</tr>
<tr>
<td>Suc + D-lactose</td>
<td>34.5</td>
<td>150.9</td>
</tr>
<tr>
<td>Suc + D-maltose</td>
<td>49.7</td>
<td>221.7</td>
</tr>
<tr>
<td>Sugar alcohols:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc + D-mannitol</td>
<td>218.9</td>
<td>694.7</td>
</tr>
<tr>
<td>Suc + D-sorbitol</td>
<td>179.5</td>
<td>322.8</td>
</tr>
<tr>
<td>Suc + D-inositol</td>
<td>112.7</td>
<td>299.3</td>
</tr>
</tbody>
</table>

+ Nitrogenase activity was measured in nmoles vial⁻¹ after 8, 16, 23 and 30 days. Oxygen if necessary was injected at 15 days.

++ Data presented at 8, 16, 23 days are extrapolated from final protein measurements after 30 days.

Values are the means of five replicates.

+++ Growth is expressed as mg protein/ml, values are means of five replicates.
Interestingly, carbon sources which supported better activity were less efficiently utilized during vegetative growth. As there was no significant difference in total protein synthesis at the cessation of activity, the carbon sources may therefore differ with regard to their ability to facilitate the synthesis and excretion of polysaccharides. This may cost the cell a part of its limited ATP pool because of microaerobic conditions. Whether carbon sources which helped to produce more polysaccharides had poor nitrogenase activity is discussed in the next chapter.

4.3.4 Effect of TCA cycle intermediates and related organic acids on nitrogenase activity.

Nitrogenase activity of strain ANU289 in basic medium containing 50mM D-arabinose and 0.05% casamino acids and different TCA cycle intermediates or related compounds (at 20mM) is shown in Table 4.3. Of the twelve organic acids tested, only succinate, pyruvate and fumarate were able to derepress or sustain derepressed nitrogenase activity. Fumarate resulted in twice as much activity compared to succinate or pyruvate. No or little activity was detected when succinate was replaced by organic acids such as acetate, citrate, glyoxylate, α-ketoglutarate, malate or malonate. Isocitrate, oxaloacetate and gluconate did not interfere with derepression of nitrogenase as all exhibited low near control levels of activity. Slightly higher activity was observed when the succinate concentration in the medium was increased from 20mM to 50mM, while 100mM resulted in cell death.

Only fumarate and pyruvate were able to replace succinate in supporting nitrogenase activity. A favourable effect of pyruvate was
**Table 4.3. Nitrogenase activity of strain ANU289 on basic media supplemented with different organic acids**

<table>
<thead>
<tr>
<th>organic acids (20mM)</th>
<th>nitrogenase activity</th>
<th>growth after 15 days (mg protein/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
<td>8 days</td>
</tr>
<tr>
<td>D-arabinose (ara)</td>
<td>20.1</td>
<td>26.9</td>
</tr>
<tr>
<td>TCA cycle intermediates:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ara + acetate</td>
<td>ND</td>
<td>5.5</td>
</tr>
<tr>
<td>ara + pyruvate</td>
<td>78.0</td>
<td>127.2</td>
</tr>
<tr>
<td>ara + citrate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ara + isocitrate</td>
<td>9.8</td>
<td>40.4</td>
</tr>
<tr>
<td>ara + ketoglutarate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ara + succinate</td>
<td>98.8</td>
<td>128.8</td>
</tr>
<tr>
<td>ara + fumarate</td>
<td>134.2</td>
<td>309.8</td>
</tr>
<tr>
<td>ara + malate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ara + oxaloacetate</td>
<td>5.5</td>
<td>20.4</td>
</tr>
<tr>
<td>other organic acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ara + malonate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ara + glyoxylate</td>
<td>ND</td>
<td>8.8</td>
</tr>
<tr>
<td>ara + gluconate</td>
<td>7.2</td>
<td>19.4</td>
</tr>
<tr>
<td>ara + succinate (50mM)</td>
<td>96.3</td>
<td>187.3</td>
</tr>
<tr>
<td>ara + succinate (100mM)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

+ Expressed as nmoles $C_2H_4 \, \text{mg}^{-1}$ protein. Values are mean of five replicates.

++ Values are means of five replicates.

ND = Not detectable.
previously shown in derepression of nitrogenase in agar slopes (Chapter 3). It is not known why these organic acids sustain good nitrogenase activity while others do not. Because these compounds are transported by a common uptake system in several rhizobia (Glenn et al. 1980) and have been implicated in energy metabolism (Skotnicki 1978), they may play a direct role in the generation of ATP in addition to the possible interaction with the sugars cited above. The isolation of mutants defective in succinate metabolism (Chapter 6) or use of uncouplers will be helpful in further analysis.

4.4 Requirement for a fixed nitrogen source for nitrogenase derepression

In contrast to other free-living nitrogen fixing organisms (e.g. Klebsiella pneumoniae), Rhizobium strains (with the exception of from Sesbania strains, (Dreyfus and Dommergues 1981, Dreyfus et al. 1983) exhibit the nitrogen fixing phenotype only in a developmental state, which is different from their normal vegetative growth state (Pankhurst and Craig 1978, Urban 1979, Pankhurst 1981, Ludwig 1983). Attempts to grow Rhizobium on its own fixed nitrogen have been difficult because derepression of nitrogenase occurred only in the presence of utilizable nitrogen source. Thus in vitro derepressed cultures are similar to bacteroids insofar as they seem to have switched off the ammonia assimilation pathway(s).

The type and amount of the particular nitrogen sources required for derepression of nitrogenase varied between strains and was dependent upon methodology and carbon sources. Glutamine was the best nitrogen source for nitrogenase activity of Rhizobium sp. 32H1 and other slow growing
rhizobia grown on agar media (Pagan et al. 1975, McComb et al. 1975, Gibson et al. 1976, Rao and Rao 1977, Pankhurst 1981), and in continuous cultures (Bergersen et al. 1976). Asparagine, histidine, urea or \((\text{NH}_4)_2\text{SO}_4\) in the medium also stimulated nitrogenase derepression. No activity was detected in the presence of alanine, cysteine, lysine, methionine or no nitrogen (Pankhurst 1981). However, in stationary (non-shaking) liquid cultures of Rhizobium sp. 32H1, casein hydrolysate and yeast extract were found to be superior to glutamate or glutamine (Evans and Keister 1976). With gluconate or succinate as the carbon source, several nitrogenous compounds, namely glutamate, glutamine, aspartate, asparagine, casamino acids, yeast extract, urea, ammonia and histidine supported nitrogenase activity in R. japonicum 61A76, USDA83 and Rhizobium sp. 32H1 grown on liquid shake cultures (Keister and Ranga Rao 1976). Valine, leucine, phenylalanine, serine and isoleucine were, however, not very effective in the same experiments.

### 4.4.1 Effect of several nitrogenous compounds on nitrogenase derepression

Since Rhizobium strains vary in terms of particular nitrogen sources required for stimulation of nitrogenase activity, all previously mentioned experiments (see Chapter 3) were done in the presence of casamino acids. Eleven alternative nitrogen sources were examined for their ability to support growth and nitrogen fixation in arabinose containing media. The results are shown in Table 4.4.

Only proline and glutamate supported higher nitrogenase activity than casamino acids in terms of rate and the maximum level of acetylene reduced. Increasing the concentration of casamino acids to 0.1% lowered the observed acetylene reduction. Histidine sustained only half the activity attained by adding casamino acids. Media containing
Table 4.4. Nitrogenase activity of strain ANU289 on basic medium supplemented with different nitrogenous compounds

<table>
<thead>
<tr>
<th>nitrogenous compound (5mM N)</th>
<th>relative* growth</th>
<th>nitrogenase activity in n moles $\text{C}_2\text{H}_4$/mg protein after**</th>
<th>8 days</th>
<th>20 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>no nitrogen (control)</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>casamino acids (0.05%)</td>
<td>++</td>
<td>131.9</td>
<td>262.5</td>
<td></td>
</tr>
<tr>
<td>casamino acids (0.1%)</td>
<td></td>
<td>27.5</td>
<td>32.3</td>
<td></td>
</tr>
<tr>
<td>glutamate</td>
<td>++</td>
<td>102.4</td>
<td>621.0</td>
<td></td>
</tr>
<tr>
<td>proline</td>
<td>NT</td>
<td>270.7</td>
<td>432.2</td>
<td></td>
</tr>
<tr>
<td>histidine</td>
<td>+</td>
<td>48.0</td>
<td>122.8</td>
<td></td>
</tr>
<tr>
<td>asparagine</td>
<td>NT</td>
<td>12.2</td>
<td>22.9</td>
<td></td>
</tr>
<tr>
<td>alanine</td>
<td>NT</td>
<td>3.9</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>glutamine</td>
<td>NT</td>
<td>17.3</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td>urea</td>
<td>+++</td>
<td>5.0</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>yeast extract (0.05%)</td>
<td>++++</td>
<td>2.0</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>$\text{KNO}_3$</td>
<td>++</td>
<td>5.5</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>$\text{KNO}_2$</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>($\text{NH}_4$)$_2\text{SO}_4$</td>
<td>++</td>
<td>5.7</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>acetamide</td>
<td>++++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>methylamine</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

* Growth was monitored at stationary phase which was attained after 5-10 days of culture. Arabinose (10mM) served as the carbon source (see Section 6.3).

** Values of nitrogenase activity are mean of five replicates, ND = not detectable, NT = not tested.
nitrogen sources such as KNO₃, KNO₂, ammonium sulphate, asparagine, alanine, glutamine, urea or yeast extract supported little or no nitrogenase activity. No relationship appeared to exist between the ability of a nitrogen source to support growth and to derepress nitrogen fixation, although in general those which promoted vigorous growth did not show any activity.

Thus, the nitrogen metabolism and regulation of nitrogenase by nitrogenous compounds in this strain showed some similarities to other strains, but there were also some key differences. Lack of activity in the absence of a fixed nitrogen source indicated that a nitrogen source is essential for derepression of in vitro nitrogenase activity in strain ANU289. This will be dealt with in detail in the next section. This characteristic is common to most of the derepressable Rhizobium strains. In agreement with the findings of Bahadur and Gour (1980), optimal nitrogenase activity was detected in the presence of either casamino acids, glutamate or proline. The latter two compounds permit the formulation of a completely defined medium.

Rhizobium strains are known to differ, however, in terms of the effect of various nitrogen compounds on derepression. For example the level of nitrate that supported nitrogenase activity in R. japonicum 61A76 (Kurz and LaRue 1975) caused inhibition of the activity in Rhizobium sp. 32H1 (Keister 1975, Scowcroft et al. 1976) and ANU289 (Table 4.7). Compounds like asparagine or ammonium sulphate which supported good activity in agar cultures of strain 32H1, did not stimulate nitrogenase expression in strain ANU289 in stationary liquid culture. The basis for such physiological differences with regard to amino acid metabolism is unknown.
4.4.2 Detection of NH$_4^+$ in the supernatant of derepressed cultures and the evidence for nitrogen fixation

A distinct feature of most in vitro derepressable rhizobia is that the majority of their fixed nitrogen has been detected as NH$_4^+$ in the supernatant, for example, as much as 94% of the $^{15}$N$_2$ fixed by R. japonicum and 80% by Rhizobium sp. 32H1 was recovered as NH$_4^+$ (O'Gara and Shanmugam 1976, Tubb 1976, Bergersen and Turner 1978). In agreement with these studies, no accumulation of ammonia in isolated bacteroids was found (Bergersen and Turner 1967, Laane et al. 1980). R. trifolii lacking ability to derepress nitrogenase metabolised L-histidine (as carbon-nitrogen source) to give rise to as high as 20µM NH$_4^+$ ml$^{-1}$ outside the cells (O'Gara and Shanmugam 1976). Similarly, histidine grown cells of R. leguminosarum can accumulate more than 100mM free ammonium in the medium via rapid equilibration across the cell membrane (Dilworth and Glenn 1982).

Strain ANU289 appeared similar to other Rhizobium strains in as far as no acetylene reduction was observed in the absence of a fixed nitrogen source. Because of the reports of liberation of ammonium to the surrounding medium in nitrogen fixing cultures of other rhizobia and the inhibition of nitrogenase activity by ammonium in this study (see previous levels), ammonium level in the supernatant was examined (Table 4.5) in rapidly agitated cultures. Keeping constant all the other conditions such as the level of glutamate, oxygen and carbon sources, when the gas phase of argon plus acetylene above derepressed cultures was replaced by dinitrogen gas, an increased level of ammonium (about 50% more than the control) was liberated to the supernatant. This could only be possible if nitrogenase was active in these cultures in reducing dinitrogen to NH$_4^+$ and excreting it to the medium. This was evident from the control, being similarly treated cells under argon plus acetylene,
Table 4.5. Ammonium concentrations in the supernatant of derepressed cultures of strain ANU289

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Ammonium Concentration</th>
<th>Acetylene Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/flask</td>
<td>nmoles/flask</td>
</tr>
<tr>
<td></td>
<td>at 30 h</td>
<td>at 60 h</td>
</tr>
<tr>
<td>I ( \text{N}_2 + 2\text{mM glutamate} )</td>
<td>225 ± 45</td>
<td>1650 ± 265</td>
</tr>
<tr>
<td>II ( \text{Argon+ acetylene + 2mM glutamate} )</td>
<td>110 ± 15</td>
<td>850 ± 130</td>
</tr>
</tbody>
</table>

This experiment was done in rapidly agitated cultures with succinate (50mM) and glucose (20mM) as carbon sources and glutamate (2mM) as nitrogen source. Each value is an average of three replicates and standard errors are given.
which showed substantial acetylene reduction but only a low level of NH$_4^+$ liberated. NH$_4^+$ detected in the control flask may have been due to glutamate metabolism via catabolic GDH and GS-GOGAT pathways operating under these conditions. Thus, this experiment demonstrated that strain ANU289 also liberates ammonium to the surrounding medium. Additionally in the absence of data for $^{15}$N$_2$ fixation, this is probably the best available evidence for N$_2$-fixation by strain ANU289 in culture.

The amount of ammonium in the supernatant was comparable to that found for 32H1 and 61A76 (O'Gara and Shanmugam 1976, Rao et al. 1982). Rao et al. (1982) detected 5-6mM ammonium in the medium while using glutamine or asparagine as sole nitrogen source in the medium. These compounds supported derepression of nitrogenase activity. In contrast formulations containing leucine, yeast extract or casamino acids neither supported nitrogenase activity nor was there noticeable liberation of ammonia. This suggested that the loss of nitrogenase activity by some strains may indeed be due to the ability of a particular strain to metabolize certain amino acids coupled with the inability to excrete ammonium. If the ammonium excretion in nitrogen-fixing cultures occurred by a different mechanism such as against a concentration gradient as in bacteroids (Laane et al. 1980) rather than a rapid equilibration as in the case of histidine grown cells (Dilworth and Glenn 1982) was not determined. Isolation of mutants unable to excrete ammonium in nitrogen-fixing (in vitro) strains may be useful in further analysis.

4.5 Molybdenum requirement for optimal nitrogenase activity

Molybdenum was thought to be essential for nitrogenase synthesis since nitrogenase comprised both the Mo-Fe protein and the Fe protein components. The role of molybdenum on derepression of nitrogenase and
Figure 4.1  Effect of Molybdenum. Molybdenum was supplied as sodium molybdate. Control (•••) received no exogenous molybdate. Derepression kinetics are plotted as nmoles ethylene per 27 ml vial (1 ml culture) over days with different levels of molybdenum 0(•••), 0.025 μM(△△△), 0.05 μM(●●●), 0.1 μM(▼▼▼), 0.25 μM(ΔΔΔ), 0.5 μM(◇◇◇) and 1.0 μM(○○○). Arabinose (50mM) and succinate 20mM (as carbon sources) and glutamate 3mM (as nitrogen source) were used.
The regulation of nitrogenase expression has been described in **N. gregaria** by Simon et al. (1980), Bode (1981) and Land (1982). Though molybdenum is supplied in the medium, the effect of molybdenum has not been verified in **N. gregaria**. Bankhurst (1981) did not find any effect of 10-25 ppm of 50 ppm fold increase in molybdenum concentration in the medium compared to the control level.

The effect of molybdenum on the depression of nitrogenase activity in the complete oxygen-deprived air-inhibitory zone in the absence of exogenously supplied molybdenum. The total activity was reduced by 50% after 17 days. Inhibition was observed with an increase in concentration up to 10 ppm molybdenum in the deoxygenated medium. Concentrations below 5 ppm of iron were also examined and the data are shown in Figure 4.1. All three iron sources had similar stimulatory effects with the optimal activity usually detected between 0.05 and 0.25 ppm Fe. In the absence of exogenously supplied iron, low (10-125 ppm) molybdenum reduction was detected.

Iron chloride normally used in the growth and deoxygenation medium showed slightly higher activity than other iron sources in strain R9201. The effect of iron was also investigated in oxygen-inhibiting inoculation (10 ppm) by Bankhurst (1981) who did not find any effect on the rate of
regulation of nitrogenase expression has been described in *Klebsiella pneumoniae* by Dixon *et al.* (1980), Eady (1981) and Kahn *et al.* (1982). Though molybdenum is supplied in the medium, the effect of molybdenum has not been verified in *Rhizobium*. Pankhurst (1981) did not find any effect of 10-, 25-, or 50-fold increases in molybdenum concentration in the medium compared to the control level.

Figure 4.1 depicts the effect of different concentrations of molybdenum on the derepression of nitrogenase activity. In the complete absence of exogenously supplied molybdenum, the total activity measured after 17 days was only one tenth of that seen with the normal concentration of molybdenum (0.1 µM). Progressively higher activity was observed with an increase in concentration up to 0.1 mM molybdenum in the derepression medium. Concentrations higher than 0.1 mM were detrimental to derepression.

4.6 Iron source and concentration effects on nitrogenase

Similar to molybdenum, an exogenous supply of iron (Fe) was essential for optimal activity. The effect of iron in three different forms such as iron chelate, iron citrate and ferric chloride in concentrations ranging from 0.01 mM to 1 mM were also examined and the data are shown in Figure 4.2. All three iron sources had similar stimulatory effects, such that optimal activity was usually detected between 0.01 and 0.05 mM Fe. In the absence of exogenously supplied iron, low (10-15% of maximal) acetylene reduction was detected.

Iron chelate normally used in the growth and derepression medium showed slightly higher activity than other iron sources in strain ANU289. The effect of iron was also investigated in cowpea strains, including 32H1 by Pankhurst (1981) who did not find any effect on the rate of
Figure 4.2 Effect of type and concentration of iron on nitrogenase activity in strain ANU289. The activity was measured after 10 days and 17 days as nmoles ethylene per 27 ml vial (1 ml culture). Iron was supplied as either iron chelate (○—○) iron citrate, (▲—▲) or ferric chloride (□—□). Activity is plotted against the concentration of iron. Arabinose (50mM) and succinate (20mM, as carbon sources) and sodium glutamate (3mM, as nitrogen source) were used.
The graph shows the relationship between iron concentration (in mM) and nmoles ethylene/vial over 10 days and 17 days. The data suggests a significant increase in ethylene production with increasing iron concentration, particularly noticeable after 17 days. The error bars indicate the variability of the data points.
development or level of nitrogenase activity by raising the concentration from 0.54 mmol/1 to 54 mmol/1 in the medium.

Thus exogenous iron, though essential for optimal expression of nitrogenase, is only necessary in small concentrations and the Fe concentration normally provided in the trace element mixture is sufficient.

4.7 Cyclic adenine nucleotides and nitrogenase expression

Cyclic AMP (cAMP) is thought to have a central role in the regulation of metabolism in many prokaryotes (Pastan and Adhya 1976). Variable effects of cAMP on derepression of nitrogenase activity have been observed. For example, in nitrogen-fixing Klebsiella aerogenes, cAMP did not appear to have a role in the regulation of nitrogenase activity, whereas a stimulatory effect was observed in another free-living bacterium viz., Azotobacter vinelandii (Lepo and Wyss 1974). Cyclic AMP was implicated on regulation of hydrogen utilization in R. japonicum (Lim and Shanmugam 1979). The latter in turn may influence nitrogenase activity. Pankhurst (1981) observed a 2- to 3- fold stimulation of nitrogenase activity in strain 32H1 and CB 756 by supplementing the medium with 1mM cAMP but not if ADP, ATP or AMP were added.

The effect of cAMP, dibutyryl - cAMP, 8-bromo - cAMP and ATP (all tested at 1mM) on established nitrogenase activity in strain ANU289 is shown in Table 4.6. In this experiment acetylene reduction was monitored for 6 days, when a low nitrogenase activity was detected. The abovementioned nucleotides were then injected aseptically through the subaseals and activity was measured for a further 6 days. As shown in Table 4.6, vials injected with cAMP reduced the activity by 50%, whereas
Table 4.6. Effect of adenine nucleotides on established nitrogenase activity in strain ANU289

<table>
<thead>
<tr>
<th>Nucleotides (1mM)</th>
<th>Nitrogenase* activity 6 days after injection</th>
<th>% of control</th>
<th>Final protein mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>167 ± 9</td>
<td>100</td>
<td>0.340</td>
</tr>
<tr>
<td>cAMP</td>
<td>89 ± 7</td>
<td>51</td>
<td>0.310</td>
</tr>
<tr>
<td>dibutyryl cAMP</td>
<td>203 ± 22</td>
<td>122</td>
<td>0.305</td>
</tr>
<tr>
<td>8br-cAMP</td>
<td>304 ± 13</td>
<td>185</td>
<td>0.315</td>
</tr>
<tr>
<td>ATP</td>
<td>160 ± 16</td>
<td>96</td>
<td>0.325</td>
</tr>
</tbody>
</table>

Nitrogenase activity was derepressed in stationary liquid cultures. Arabinose (50mM) succinate (20mM) (as carbon sources), casamino acids 0.05% as nitrogen source.

* activity was measured as nmol C₂H₄mg⁻¹ protein using 6 replicates and is expressed as the increase between the sixth day and twelfth day reading. Standard errors are shown.
8Br cAMP increased activity by nearly 85% over the unsupplemented control. In contrast, ATP had no significant effect, presumably because of uptake difficulties in this in vivo system. A moderate stimulation over control activity was observed when the dibutyryl ester of cAMP (dibutyryl-cAMP) was used. This was known to be more effective in E. coli due to increased uptake.

Thus, of all cyclic nucleotides tested, 8-bromo-cyclic AMP (an analogue of cAMP), claimed by Wood and Braun (1973) to prevent the breakdown of cAMP in plant and animal tissue and to regulate cell division inexplicably increased nitrogenase activity significantly.

4.3 Summary

The studies in Chapter 4 thus involved primarily the ability of strain ANU289 to grow and to derepress nitrogenase activity on a range of sugars, organic acids and nitrogenous compounds. The effect of different concentrations of molybdenum and iron (supplied in various forms) and the effect of cyclic nucleotides on established nitrogenase activity were also examined.

This Rhizobium strain was capable of growth on all carbon sources tested, except for disaccharides such as sucrose, maltose and lactose, which supported poor growth. Compared to previously examined strains, it had a different spectrum of sugar requirements for derepression of nitrogenase activity in as far as hexose sugars/disaccharides/sugar alcohols in combination with succinate stimulated better nitrogenase activity. Thus, strain ANU289 was a typical slow grower in respect of growth, but was different in terms of requirements for nitrogenase expression.
Growth and nitrogenase expression of strain ANU289 in various organic acids demonstrated that it was not different from other 'cowpea Rhizobium' strains in this regard. It appeared that organic acid-metabolism, rather than sugar metabolism, played a major role in derepression.

Strain ANU289 also utilized all the nitrogenous compounds tested except methylamine. Only three of these, namely glutamate, proline and casamino acids, supported optimal nitrogenase activity. More significantly, ammonium and glutamine both prevented expression of this activity, but this needed further verification (see Chapter 6).

Molybdenum and iron effects were examined. The concentration at which molybdenum is present in the culture medium is appropriate for obtaining optimal nitrogenase activity. Although only trace amounts of molybdenum were required, its absence caused a severe decline in activity. Similarly with iron between 0.01 and 0.05mM concentration, irrespective of the source, the strain sustained good nitrogenase activity.

Finally, four cyclic nucleotides were examined for their effect on nitrogenase expression after injecting them at appropriate concentrations into cultures with established nitrogenase activity. The results were quite contrary to those expected. Cyclic AMP reduced the activity by 50%; in contrast 8-bromo-cyclic AMP (an analogue of cAMP) significantly increased nitrogenase activity.

Thus overall, in some respects, strain ANU289 was similar to other 'cowpea rhizobia' in requirements for nitrogenase activity whereas in some other respects it was different. The altered carbon requirement and cAMP effects conclusively demonstrated that strain ANU289 was different from other 'cowpea rhizobia' and particularly well characterised strain 32H1. This may have a significance in view of their differential ability (see Section 3.1) to fix nitrogen in Parasponia. The other notable distinction
was the extreme sensitivity of nitrogenase activity in strain ANU289 to nitrogenous compounds such as glutamine and ammonia as opposed to strain 32H1. These studies provided a basis for a detailed analysis of carbon and nitrogen metabolism in strain ANU289.
CHAPTER-5

ROLE

OF

CARBON AND ENERGY METABOLISM

IN DEREPRESSION OF NITROGENASE ACTIVITY:

STUDIES USING MUTANT ANALYSES
5. ROLE OF CARBON AND ENERGY METABOLISM IN DEREPRESSION OF NITROGENASE ACTIVITY: STUDIES USING MUTANT ANALYSES

5.1 Preface

This chapter reports on analyses of genetic conditionality in carbon and energy metabolism and its effects on nitrogenase derepression and activity. Three major systems are open to genetic investigation. They are: (1) conditionality for exopolysaccharide (EPS) production, (2) succinate utilisation (uptake and metabolism) and (3) the uptake hydrogenase and hydrogen metabolism. These aspects form three distinct sections of this chapter. Additionally a description of the methods pertinent for the chapter is included (see Section 5.3).

5.2 Mechanism of regulation by carbon sources

The results presented in Chapter 3, coupled with the several other previous observations (see e.g. Gibson et al. 1976, Keister and Ranga Rao 1976, Pankhurst 1981) confirmed that provision of an appropriate carbon source in derepression media is a necessary pre-requisite for expression of nitrogenase activity in rhizobia. The precise role, however, of a carbon source in nitrogenase derepression was not understood.

In the absence of evidence to the contrary, it is assumed that the establishment of nitrogenase activity in rhizobia at the molecular level, similar to other free-living bacteria, involves a number of steps including the biosynthesis of the constituent polypeptides of the enzyme, the incorporation of co-factors, the functioning of an electron transport system to nitrogenase and the availability of sufficient energy. Using an experimental approach that allows the quantitative determination of the de novo biosynthesis of the constituent polypeptides of nitrogenase in free-living R. japonicum, the amount of synthesis was shown to be dependent upon the carbon source used (Scott et al. 1979). Cells grown
on mannitol or glycerol produced only trace amounts of the nitrogenase polypeptides, whereas gluconate supported maximal synthesis and whole cell nitrogenase activity.

Since exogenously supplied carbon is a major source of ATP and reducing equivalents in the cell, various carbon sources may exert their effects by altering the general metabolism of the cell (Keister and Ranga Rao 1976), thus differentially influencing the level of potential activity, after the nitrogenase proteins are synthesized. Some of the factors which have been implicated in the rapid modulation of nitrogenase activity in bacteroids are the ATP/ADP ratio (Appleby et al. 1975, Laane et al. 1978) and the maintenance of a sufficiently low oxygen concentration and membrane potential (Laane et al. 1978), the latter two being important in the supply of reducing equivalents.

Thus, carbon sources may regulate nitrogenase activity either directly (nitrogenase polypeptide synthesis) or indirectly (modulation of activity). The relative importance of these phenomena probably depends on apparently interrelated factors, such as (1) differences between strains with regard to their carbon source requirement(s) for optimal growth and nitrogenase expression (see Chapter 4.3), (2) pathways of carbon metabolism operating under these microaerobic nitrogen-fixing conditions and the energy yield associated with these pathways, (3) the extent of intermediary metabolism, for example extracellular polysaccharide synthesis and (4) the range of other proteins synthesized under these conditions concomitant with nitrogenase and particularly those required for nitrogenase activity and perhaps assimilation and/or export of fixed nitrogen. It is known that under microaerobic conditions the cell becomes more specialized through the selective synthesis of several proteins (Scott et al. 1979).
In view of the availability of two isogenic Parasponia-Rhizobium strains ANU288 (mucoid) and ANU289 (non-mucoid) differing in their ability to produce EPS on mannitol containing media and high acetylene reduction activity of strain ANU289 in the presence of mannitol (see Section 4.3.3), it was of value to examine the relationship between EPS synthesis and nitrogenase activity in these strains. Thus, it was possible to use a genetic conditionality for EPS production on the same carbon source (i.e. mannitol) as a means of dissecting the interrelation between these two metabolic processes.

Moreover, the effect of varying the carbon sources and its concomitant alteration of EPS levels allowed the analysis of relationships between EPS synthesis, growth (in terms of total protein), respiration (measured as oxygen consumption), as well as in vitro nitrogenase activity. The data are presented in Section 5.4.

Similarly, the role of succinate in nitrogenase derepression was determined employing a genetic approach. Mutants of strain ANU289, defective in succinate metabolism were isolated (see Section 5.5). Using these mutants the role of succinate in nitrogen fixation was evaluated.

Furthermore, hydrogen uptake negative mutants of strain ANU289 were isolated and analysed for their nitrogenase phenotype both in planta and in vitro (Section 5.6).

5.3 Methods

The techniques such as growth tests on plates, and liquid cultures, protein determination, contamination tests, nodulation on siratro, in planta nitrogenase activity measurements and bacteroid isolation
procedures used in experiments in this chapter were essentially similar to those described in Chapter 2.3. For experiments dealing with derepression of nitrogenase activity, the stationary liquid culture approach (Section 2.3.2) was followed.

5.4 Extracellular polysaccharide synthesis and nitrogenase activity

5.4.1 Background

Gum or mucoid matter (also called extracellular polysaccharide, EPS) is characteristic of aerobic nitrogen-fixing bacteria particularly among Azotobacteriaceae (Postgate 1971). A positive correlation between EPS synthesis and nitrogen fixation was shown in many strains of Beijerinckia (Barooah and Sen 1964). It was suggested that EPS produced on the culture plate may have impeded oxygen transfer in bacterial cultures (Postgate 1971) and thereby affect the level of nitrogenase activity. In slow-growing Rhizobium strain 32H1, Kurz and LaRue (1975) related the observations of increased acetylene reduction to EPS production. Since succinate was previously reported to promote the synthesis of polysaccharide (Dudman 1964) as well as nitrogenase activity (Gibson et al. 1976) in rhizobia, a positive correlation seemed apparent. Pankhurst and Craig (1978) suggested that the oxygen gradient formed in soft agar cultures was due to polysaccharide production by the Rhizobium on the surface. Due to the presence of an unusual sugar (6-deoxy-L-talose) as a major component of EPS in two easily derepressable 'cowpea strains' namely 32H1 and CB756 (Kennedy 1978), it seemed possible that the presence of unusual sugars in the EPS of these bacteria could be related to their ability to develop nitrogenase. Kennedy and Pankhurst (1978) examined the EPS composition and in vitro nitrogenase activity in 10 different Rhizobium strains belonging to...
Rhizobium sp. as well as Rhizobium loti and found no correlation. Wilcockson (1977) first observed that slime did not 'protect' nitrogenase from oxygen damage in Klebsiella. Later a poor correlation between EPS production and nitrogenase activity in two unrelated R. japonicum strains was reported (Wilcockson and Werner 1978).

In liquid cultures, however, the Rhizobium strains which have been derepressed for nitrogenase activity are those which were empirically observed to produce a low amount of EPS (Keister and Evans 1976, Keister and Ranga Rao 1976). Using 20 different R. japonicum strains and two different carbon sources, Agarwal and Keister (1982) demonstrated a definite negative correlation between EPS synthesis and nitrogen fixation. Thus, in the Rhizobium literature alone there are arguments suggesting either a positive or negative correlation between EPS production and nitrogenase activity.

5.4.2 Isolation of strain ANU289 and ANU288

Two phenotypically distinct colony types were identified from Parasponia andersonii nodule isolate CP283, when grown on BMM medium. The rough opaque colony type was called strain ANU289 (Fig. 5.1) and the smooth concentric type (an opaque centre surrounded by translucent extracellular polysaccharides) was called strain ANU288 (Fig. 5.1). Except for mucoidy characteristics on mannitol, strains ANU288 and ANU289 are believed to be isogenic for the following reasons: (1) both strains exhibit similar levels of spontaneous resistance to the antibiotics rifampicin, trimethoprim, carbenicillin, gentamycin, erythromycin, neomycin and tetracycline (B.G. Rolfe, pers. comm.); (2) both strains effectively nodulate the tropical legume siratro and non-legume Parasponia rigida; (3) serotyping results show that both strains belong to the same serogroup (M. Trinick, pers. comm.); and (4) both strains ANU288 and
Figure 5.1. Colony dimorphism of Parasponia nodule isolate CP283 on mannitol containing BMM plates. The mucoid phenotype with typical smooth concentric an opaque centre surrounded by translucent extra-cellular polysaccharides colony morphology (A and B) was named as strain ANU288. The rough opaque colony type was named as strain ANU289 (C and D).
ANU289 exhibited identical restriction fragment patterns after their total DNA was restricted with the endonucleases EcoRI and Hind III (K. Scott, pers. comm.).

5.4.3 Growth and derepression kinetics on liquid media

Although strains ANU288 and ANU289 exhibited distinct growth patterns on agar plates with mannitol (Fig. 5.1), it was difficult to assess their growth rates. Furthermore, strain ANU288 was derepressed using D-arabinose and succinate as carbon sources (Section 3.4.1) but not mannitol. On account of the isogenic behaviour, except for growth on mannitol containing plates, further characterization of strains ANU288 and ANU289 was necessary. This section reports the initial studies dealing with growth and derepression of these strains in liquid media containing mannitol.

The growth kinetics of strain ANU288 and ANU289 in liquid RGM30M and RGM30A (Chapter 2) media containing D-mannitol (10mM) and D-arabinose (10mM) respectively are shown in Fig. 5.2. Strain ANU288 which was capable of producing large amounts of polysaccharide on BMM plates containing mannitol (section 5.3.2), exhibited a rapid increase in turbidity 5 days after inoculation compared to ANU289. A parallel increase in turbidity was noted for both strains on media containing D-arabinose. However, culture samples withdrawn at regular intervals indicated a similar increase in cell number for both strains and media. The production of EPS was maximal in the logarithmic growth phase. Parent strain CP283, from which strain ANU288 and ANU289 were isolated showed an intermediate growth curve.

Figure 5.3 shows the comparative derepression kinetics for nitrogenase activity in strains ANU288 and ANU289 in a medium containing D-mannitol (50mM) and succinate (50mM) as carbon sources. Strain ANU288
Figure 5.2. Growth kinetics of strains ANU288 and ANU289 as well as the parent strain CP283 in liquid media (RGM30) containing mannitol (10mM) as a carbon source and glutamate (10mM) as a nitrogen source. ○ ○, strain ANU288; △ △, ANU289 and □, CP 283. Growth of strains ANU288 and ANU289 in a similar medium but arabinose (10mM) instead of mannitol is shown by broken lines (△-----△). Growth was measured as Klett units (see section 2.3.1.).
Figure 5.3. Comparative derepression kinetics of nitrogenase activity of strains ANU288 and ANU289. The derepression medium contained 50mM mannitol, 50mM succinate, and 3mM glutamate. The gas phase consisted of 5% $O_2$, 4% acetylene, 91% helium (stationary culture). The activity was measured as nmoles ethylene mg$^{-1}$ protein. The values are average of 5 replicates. The error bar represents the average standard error.
The differential ability of growth and ethylene production in the presence of mannitol was also investigated in two strains of P. pisi. The results indicated a strong correlation between these strains with regard to both activities and also between both activities and mannitol concentration. It is possible to maintain AN69 and AN88 strains without mannitol in media containing normal concentrations of carbohydrates. This is particularly true in media containing sucrose. However, mannitol is required if the strains are grown over prolonged storage or maintenance in agar slants. Interestingly, the occurrence of such mutants has been previously described in Leuconostoc mesenteroides (Ott et al., 1961). A total of 96 auxotroph mutants were obtained from strain AN69 at a frequency of $3 \times 10^{-8}$. A total of
showed only one third of the maximal activity of strain ANU289. Acetylene reduction activity in the presence of mannitol was four times higher than activity in the presence of succinate alone. This was consistent with mannitol effects seen in Chapter 4. These results, coupled with the studies of mutant ANU289-M-1, clearly indicated a negative correlation between these strains with regard to both EPS synthesis and nitrogenase activity.

5.4.4 Mutational analysis of mucoidy characteristics of ANU289

The differential ability for growth and derepression of nitrogenase activity of two supposedly isogenic strains provided incentive for further studies in view of the possible correlation between EPS synthesis and nitrogenase activity. It is possible that strain ANU288 arose spontaneously from ANU289 with the ability to make these polysaccharides during prolonged storage on yeast-mannitol-agar slants (Trinick 1980). Interestingly, the occurrence of such spontaneous mutations during storage resulting in new polysaccharide variants was previously reported in other rhizobia (Ghai et al. 1981). If strain ANU288 is a spontaneous mutant of strain ANU289, ANU288-like isolates can be obtained by mutagenising ANU289. Such attempts are delineated in this section.

The possibility that strain ANU288 arose from strain ANU289 was tested by mutagenising ANU289 cells (with NTG, 200 mg/l for 6 hours) and plating onto mannitol containing media. Mucoid (muc+) colonies were obtained from strain ANU289 at a frequency of $2 \times 10^{-4}$. A total of 5 muc+ mutants were selected; of these one (ANU289-M-1) was chosen for further study.

A comparison of growth, EPS production and acetylene reduction in Parasponia-Rhizobium strains ANU288, ANU289 and the EPS mutant ANU-289-M-1 is presented in Table 5.1. The mutant had a growth pattern similar to that of strain ANU288 in liquid media containing arabinose, mannitol or
Table 5.1. Comparison of aerobic growth and EPS production and acetylene reduction in *Parasponia-Rhizobium* strains ANU288, ANU289 and the mutant ANU289-M-1

<table>
<thead>
<tr>
<th>Strains</th>
<th>In aerobic culture with mannitol</th>
<th>Microaerobic culture with mannitol + succinate acetylene/reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>EPS produced</td>
</tr>
<tr>
<td>ANU289</td>
<td>+</td>
<td>74 (100)</td>
</tr>
<tr>
<td>ANU288</td>
<td>+++</td>
<td>273 (372)</td>
</tr>
<tr>
<td>ANU289-M-1</td>
<td>+++</td>
<td>154 (210)</td>
</tr>
</tbody>
</table>

Growth was determined by Klett reading (see Fig. 5.2.). EPS was measured as µg glucose mg protein⁻¹ average of two independent experiments. Values in parentheses are standardised percentages using ANU289 as 100%. Acetylene reduction was measured as total nmoles of ethylene produced per mg protein after 15 days. The average standard error was ± 73.
gluconate (data not shown). However, it was only intermediate in EPS producing ability (falling in between ANU289 and ANU288). Acetylene reduction characteristics were similar to those of strain ANU288 except that higher activity was obtained in the mutant. Despite the intermediate EPS phenotype, the inverse relationship between EPS production and nitrogenase activity remained clear.

Although EPS production has an adverse effect on in vitro nitrogenase synthesis and activity, the same was not true for in planta nitrogenase activity. The nodule environment was more favourable for strain ANU288 than for ANU289 (G. Bender pers. comm.). For example, nodules initiated by a 100:1 mixture of ANU289:ANU288 (pre-cultured on BM), were found to be solely occupied by strain ANU288 at the time of nitrogen fixation. There is a great deal of controversy regarding the importance of EPS in the ability of Rhizobium strains to form nitrogen-fixing nodules in host plants (Sanders et al. 1978, Napoli and Albersheim 1980, Chakravorty et al. 1982). These findings lead to the conclusion that EPS synthesis is perhaps necessary for the development of nitrogen-fixing nodules and thus are in agreement with those of Chakravorty et al. (1982).

5.4.5 Strain differences used to study the relationship between growth (protein content), EPS synthesis and oxygen consumption in aerobic and microaerobic liquid cultures

The genetic conditionality in respect of EPS synthesis between strains ANU288 (or the EPS mutant ANU289-M-1) were helpful in demonstrating an inverse relationship between EPS synthesis and in vitro nitrogenase activity. EPS synthesis in the previous study was measured under aerobic conditions. The spectrum of EPS synthesis may, however,
change under nitrogen-fixing conditions. Thus, a comparison between strains ANU288 and ANU289 in both aerobic and nitrogen-fixing conditions was warranted. Five carbon sources, other than mannitol were used in this study. In view of the observed relationship of growth (this study) as well as oxygen consumption (Agarwal and Keister 1982) to *in vitro* nitrogenase activity, an analysis of growth and oxygen consumption was included while studying the correlation between nitrogenase activity and EPS synthesis. The data are presented in Table 5.2.

Under aerobic conditions the differences between strains ANU288 and ANU289 were less pronounced on sorbitol or gluconate. In fructose or arabinose containing media, ANU288 exhibited a higher rate of oxygen consumption and EPS synthesis but a lesser increase in protein concentration than strain ANU289. In the presence of sucrose, both strains exhibited higher rates of oxygen consumption and less protein synthesis. Strain ANU288 produced more EPS than strain ANU289 in the presence of sucrose. When compared with the no additional carbon control, some sugars had overall repressive effects on growth. These results differ from those of growth on agar plates containing the same sugars and 2mM (NH₄)₂SO₄ but no succinate (G. Bender, pers. comm.). On such agar plates both mannitol and fructose resulted in smooth concentric type colonies of strain ANU288, but not of strain ANU289. All other carbon sources supported similar growth patterns being either mucoid (as in the case of gluconate) or non-mucoid (as with arabinose, sorbitol and sucrose) in both strains.

The differences observed between aerobic liquid cultures and agar plate-studies may be due to the presence of succinate in the former. Succinate is known to modulate uptake and metabolism of sugars. Though such examples are not known in 'cowpea rhizobia', they were recently demonstrated in another slow-growing species, namely *R. japonicum*
Table 5.2. Relationship between growth (in protein), EPS synthesis, oxygen consumption and acetylene reduction in Parasponia-Rhizobium strains ANU288 and ANU289 in aerobic and microaerobic conditions.

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Experimental conditions</th>
<th>Increase in protein ANU288</th>
<th>EPS ANU288</th>
<th>EPS ANU289</th>
<th>O₂ consumption ANU288</th>
<th>O₂ consumption ANU289</th>
<th>Acetylene reduction ANU288</th>
<th>Acetylene reduction ANU289</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-arabinose + succ</td>
<td>aerobic</td>
<td>.363</td>
<td>214</td>
<td>114</td>
<td>96</td>
<td>19</td>
<td>290</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>microaerobic</td>
<td>.240</td>
<td>43</td>
<td>44</td>
<td>5</td>
<td>13</td>
<td>687</td>
<td>1779</td>
</tr>
<tr>
<td>D-fructose + succ</td>
<td>aerobic</td>
<td>.205</td>
<td>296</td>
<td>170</td>
<td>792</td>
<td>38</td>
<td>1084</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>microaerobic</td>
<td>.200</td>
<td>32</td>
<td>46</td>
<td>4</td>
<td>11</td>
<td>687</td>
<td>1779</td>
</tr>
<tr>
<td>D-sorbitol + succ</td>
<td>aerobic</td>
<td>.345</td>
<td>103</td>
<td>161</td>
<td>153</td>
<td>96</td>
<td>687</td>
<td>1779</td>
</tr>
<tr>
<td></td>
<td>microaerobic</td>
<td>.160</td>
<td>36</td>
<td>20</td>
<td>21</td>
<td>12</td>
<td>687</td>
<td>1779</td>
</tr>
<tr>
<td>D-sucrose + succ</td>
<td>aerobic</td>
<td>.120</td>
<td>227</td>
<td>161</td>
<td>326</td>
<td>840</td>
<td>560</td>
<td>1335</td>
</tr>
<tr>
<td></td>
<td>microaerobic</td>
<td>.240</td>
<td>38</td>
<td>26</td>
<td>14</td>
<td>35</td>
<td>560</td>
<td>1335</td>
</tr>
<tr>
<td>Gluconate + succ</td>
<td>aerobic</td>
<td>.437</td>
<td>176</td>
<td>176</td>
<td>96</td>
<td>96</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>microaerobic</td>
<td>.380</td>
<td>65</td>
<td>70</td>
<td>8</td>
<td>7</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>Succinate (succ)</td>
<td>aerobic</td>
<td>.200</td>
<td>98</td>
<td>104</td>
<td>96</td>
<td>75</td>
<td>211</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>microaerobic</td>
<td>.100</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>211</td>
<td>327</td>
</tr>
</tbody>
</table>

Carbon sources included both sugar (at a concentration of 50mM) and succinate (20mM). Glutamate (3 mM) was present as a nitrogen source. Aerobic (air in the gas phase) studies were done in 250 ml klett sidearm flasks with cotton plugs and shaken at 180 rpm. Microaerobic (5% O₂ v/v) experiments were done following stationary liquid culture technique. Increase in protein was measured as mg ml⁻¹. EPS production was measured as grams of glucose mg⁻¹ protein. O₂ consumption was measured either as moles mg⁻¹ day⁻¹ (aerobic cultures) or n moles mg⁻¹ day⁻¹ (microaerobic cultures). Acetylene reduction was measured as nmoles of ethylene mg⁻¹ protein after 15d incubation.
Of particular interest was sucrose, on which strain ANU289 was unable to grow as a sole carbon source (Chapter 4.3). As shown in Table 5.2, in both strains the rates of oxygen consumption was exceptionally high on sucrose in conjunction with succinate and glutamate, as were the EPS values. As to how sucrose, which is not utilized by strain ANU289, can have an effect on oxygen consumption and nitrogenase activity, (see Chapter 4.3) is totally unclear.

Under nitrogen-fixing microaerobic conditions, the spectrum of protein and EPS synthesis and the rates of oxygen consumption were different from those under aerobic conditions (Table 5.2). The growth measured as protein, varied depending on the carbon source present. No definite correlation was apparent between the increase in protein content and acetylene reduction. For example, on fructose, both strain ANU288 and ANU289 showed a similar protein increase, but high acetylene reduction was observed only in strain ANU288. Similar to that of mannitol, an inverse relationship between EPS production and nitrogenase activity was observed in media containing fructose, sorbitol and sucrose between strains ANU288 and ANU289. Arabinose and gluconate stimulated high EPS and protein synthesis but less nitrogenase activity in both of these strains. These results are in agreement with the previously observed negative correlation between EPS synthesis and nitrogenase activity.

No clear cut correlation between oxygen consumption and nitrogenase activity could be demonstrated in either strain. Interestingly, on sucrose the oxygen consumption rates were high in microaerobic conditions (similar to aerobic conditions) in strain ANU289. This culture also showed high nitrogenase activity.
This study not only confirmed the difference between strains ANU288 and ANU289 with respect to carbon metabolism and EPS synthesis, but also indicated that carbon compounds play a major role in determining the level of EPS synthesis and nitrogenous activity.

5.4.6 Use of only one strain (ANU289) to study the effect of a wide range of carbon sources on EPS production

In Chapter 4, growth of strain ANU289 on different carbon sources was described. The results obtained in Section 5.4.5 showed that the strains ANU288 and ANU289 differed from one another in their ability to produce exopolysaccharide, therefore differed with regard to acetylene reduction. It was not understood what effect carbon sources had on the establishment of the correlation. Thus, keeping the bacterial strain constant, i.e. using strain ANU289 alone (thus avoiding any genetic differences) the effect of several other carbon sources was examined with regard to EPS synthesis, oxygen consumption and acetylene reduction. The results are presented in Table 5.3.

Differential derepression of nitrogenase was observed in media containing different carbon sources. A reciprocal relationship between EPS synthesis and nitrogenase activity was observed in general (see the scatter diagram Fig 5.4). However, oxygen consumption was variable and specifically dependent upon the carbon sources used. Scatter diagrams correlating $O_2$ consumption with acetylene reduction as well as EPS production indicated that there was no direct correlation between these parameters. Additionally (data analysed but figures not shown) there was no correlation between these parameters and with protein content. In other words, the precise relationships between all these parameters were highly complex. Even if one demonstrated a statistically significant
Table 5.3. Differential effects of carbon sources on EPS production, oxygen consumption and acetylene reduction

<table>
<thead>
<tr>
<th>carbon sources</th>
<th>total protein (mg/ml)</th>
<th>EPS glucose/mg protein</th>
<th>O₂ consumption (nmoles/day/mg protein)</th>
<th>acetylene reduction (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars (50mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>succinate 20mM(suc)</td>
<td>0.17</td>
<td>5</td>
<td>7.3</td>
<td>427</td>
</tr>
<tr>
<td>suc + D-galactose</td>
<td>0.23</td>
<td>135</td>
<td>10.0</td>
<td>364</td>
</tr>
<tr>
<td>suc + D-glucose</td>
<td>0.31</td>
<td>87</td>
<td>4.2</td>
<td>566</td>
</tr>
<tr>
<td>suc + D-lactose</td>
<td>0.29</td>
<td>71</td>
<td>9.3</td>
<td>596</td>
</tr>
<tr>
<td>suc + D-maltose</td>
<td>0.28</td>
<td>79</td>
<td>4.3</td>
<td>972</td>
</tr>
<tr>
<td>suc + D-mannose</td>
<td>0.25</td>
<td>40</td>
<td>4.2</td>
<td>1296</td>
</tr>
<tr>
<td>suc + D-xylose</td>
<td>0.28</td>
<td>164</td>
<td>12.0</td>
<td>270</td>
</tr>
<tr>
<td>suc + D-inositol</td>
<td>0.23</td>
<td>30</td>
<td>7.4</td>
<td>1197</td>
</tr>
<tr>
<td>organic acids (20mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-arabinose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50mM (ara)</td>
<td>0.23</td>
<td>216</td>
<td>16.8</td>
<td>35</td>
</tr>
<tr>
<td>ara + acetate</td>
<td>0.12</td>
<td>150</td>
<td>7.1</td>
<td>70</td>
</tr>
<tr>
<td>ara + citrate</td>
<td>0.05</td>
<td>260</td>
<td>8.0</td>
<td>ND</td>
</tr>
<tr>
<td>ara + fumarate</td>
<td>0.20</td>
<td>116</td>
<td>10.5</td>
<td>442</td>
</tr>
<tr>
<td>ara + ketoglutarate</td>
<td>0.01</td>
<td>166</td>
<td>3.3</td>
<td>138</td>
</tr>
<tr>
<td>ara + malate</td>
<td>0.01</td>
<td>250</td>
<td>5.0</td>
<td>59</td>
</tr>
<tr>
<td>ara + malonate</td>
<td>0.05</td>
<td>200</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td>ara + succinate (50mM)</td>
<td>0.15</td>
<td>62</td>
<td>11.7</td>
<td>889</td>
</tr>
</tbody>
</table>
Figure 5.4. Scatter diagrams describing relationship of acetylene reduction with oxygen consumption (upper), growth as measured by protein synthesis (middle) and EPS synthesis (bottom). The correlation co-efficients are shown. Numerical data are presented in Table 5.3.
These acetylene reduction data are in agreement with those observed previously in Chapter 5. The differential utilization of nitrogenase activity by various fraction sources appears to be due to their effects on producing varying amounts of EPS under the nitrogen-fixing conditions. Sucrose alone, in contrast to the other fractions, did not produce more polyamine nitrogenase activity.

Thus a study in this acetylene reduction indicated that EPS production and nitrogenase activity were reciprocally related. EPS production can vary depending on strain and/or carbon source being used in the experiment. This study determines the levels of nitrogenase expression. In contrast, acetylene reduction may not be exponential. It appears that the total energy output as measured by respiration (as well as consumption and partitioning into ammonia and EPS) may be partitioned into some unknown, undetermined (in terms of total protein). EPS synthesis may arise from the fact that acetylene reduction integrates effects of (a) the repression of nitrogenase and (b) the maintenance of nitrogenase activity. There is a temporal differentiation of these two processes because of the fact. Acetylene reduction the EPS levels may affect the appearance of activity whether the growth versus oxygen consumption rates are determined in the MR/ABD initial stages or control the initial derepression and differentiation into a nitrogen-fixing state.
relationship between any two of the parameters, it might be fortuitous. This may not be the case for acetylene reduction and nitrogenase activity.

These acetylene reduction data are in agreement with those observed previously in Chapter 4. The differential stimulation of nitrogenase activity by various carbon sources appears to be due to their effects on producing varying amounts of EPS under these nitrogen-fixing conditions. Succinate alone, in microaerobic as compared to aerobic conditions did not produce more polysaccharide and supported high nitrogenase activity. This was also observed by Wilcockson and Werner (1978).

Thus studies in this section clearly indicated that EPS production and nitrogenase activity are reciprocally related. EPS production can vary depending on the strain and/or carbon source being used in the experiment. This in turn determines the level of nitrogenase expression. In contrast an absolute correlation between $O_2$ consumption and nitrogenase activity could not be established. It appears that the total energy efflux as measured by respiration ($O_2$ consumption) can be partitioned into components such as general growth (in terms of total protein), EPS synthesis and nitrogenase activity. Part of the complexity may arise from the fact that acetylene reduction data integrate effects on (a) the derepression of nitrogenase and (b) the maintenance of nitrogenase activity. Thus, a temporal differentiation of these two processes becomes distinct. Admittedly speculative, the EPS levels may affect the maintenance of activity whereas the growth versus oxygen consumption rate (perhaps via the ATP/ADP ratio) may control the initial derepression and differentiation into the nitrogen-fixing state.
5.5 Nitrogenase activity in mutants defective in succinate utilization

In an effective legume-Rhizobium symbiosis, the bacteroids within the nodules are entirely dependent for their energy source on photosynthate supplied by the host plants. Bacteroids consume about 3-7 milligrams of carbohydrate per milligram of nitrogen fixed in actively developing nodules (Gibson 1966, Mahon 1979, Ryle et al. 1979). However, the nature of the carbon substrate(s) used by bacteroids within the nodule has not been clearly established. Evidence suggests that sucrose is transported to the nodules where the alkaline invertase activity is higher than in the surrounding roots (Kidby 1966, Robertson and Taylor 1973, Singh et al. 1980). When host plants were fed with \(^{14}\)CO\(_2\), over 60% of the label was found as glucose and fructose in French bean or soybean nodules, whereas in bean nodules it was primarily found as a sucrose (Bach et al. 1958, Lawrie and Wheeler 1975, Antoniw and Sprent 1978). Isolated soybean bacteroids did not oxidise fructose, glucose, sucrose and other hexoses (Tazimura and Meguro 1960) while sucrose was shown not to support nitrogen fixation (Bergersen and Turner 1967). Ronson and Primrose (1979) obtained a number of R. trifolii mutants that were defective in hexose uptake or metabolism. These were able to form nitrogen-fixing root nodules thus indicating no significant role of these compounds in root nodules. In contrast, stimulation of nitrogen fixation by sucrose in nodule slices of soybean was shown (Bach et al. 1958). Sucrose or glucose alone were able to provide energy and support nitrogen fixation in bacteroids of French bean, soybean and pea at low oxygen tension (Rigaud et al. 1973, Trinchant et al. 1981). Additionally a phosphoglucose isomerase mutant of R. meliloti was found to be partially effective. Thus, the role these carbon compounds play in supporting nitrogen fixation is unresolved. It may be too optimistic to hope for one unique answer for all Rhizobium species,
especially in view of the major evolutionary differences which seem to exist between the *Rhizobium* (the fast growing species in general) and the newly described *Bradyrhizobium* (the slow growing species).

The $^{14}$C labelled organic acid fraction comprised up to 20% of the total label in the nodule (Lawrie and Wheeler 1975), with malate being the most abundant acid. Stumpf and Burris (1979) quantified the organic acid content in soybean and found that the nodule tissue contained three times more succinate, six times more fumarate and two times more malate than root tissue. Succinate, fumarate and other TCA cycle intermediates were readily oxidised by isolated soybean bacteroids (Tazimura and Meguro 1960); the former two also stimulated nitrogenase activity in such bacteroids (Bergersen and Turner 1967, Houwaard 1979). Reports by both Kurz and LaRue (1977) and Stovall and Cole (1978) indicated the presence of a functional TCA cycle in bacteroids isolated from peas and soybeans. Additionally, *Rhizobium* mutants defective in TCA cycle-enzymes such as the 2-oxo-glutarate dehydrogenase deficient mutant of *R. meliloti* (Duncan and Fraenkel 1979), the succinate permease deficient mutant of *R. trifolii* (Ronson et al. 1981) and the succinate resistant mutants (characterised by decreased rates of succinate uptake and metabolism) of *R. leguminosarum* (Glenn and Brewin 1981) were able to nodulate (nod$^+$) but unable to fix nitrogen (fix$^-)$.

In Chapter 4, it was shown that succinate played a major role in *in vitro* nitrogenase derepression by strain ANU289. The ability of succinate to support nitrogenase activity in free-living cultures of other *Rhizobium* strains has been observed by Gibson et al. (1976), Keister and Kanga Rao (1976), Pankhurst (1981) and many others. Earlier in this chapter (see Section 5.4), the ability of succinate to interact with other carbon sources and thus modulate both EPS synthesis and nitrogenase
activity was described. To define further the effects of succinate on derepression of nitrogenase both in vitro and in planta, NTG mutagenesis of strain ANU289 was undertaken to isolate mutants defective in succinate metabolism. The characterisation of these mutants in terms of their nitrogen-fixing capacity is described in the following section.

5.5.1 Isolation of mutants and growth characteristics

Sufficient energy for cell growth can be derived from succinate by oxidative phosphorylation. Hence mutants uncoupled in oxidative phosphorylation (unc) cannot grow on succinate as a carbon source alone. In *E. coli* (Sasaraman et al. 1970, Kanner and Gutnick 1972) unc mutants have been isolated by their resistance to the antibiotic neomycin, which allows direct selection. The same technique was successfully applied in *R. trifolii* (Skotnicki 1978) to isolate unc mutants. Therefore, this technique was chosen to isolate mutants in strain ANU289 defective in succinate uptake or metabolism. These mutants were then used to examine the role of succinate in nitrogenase derepression.

For chemical mutagenesis, it was necessary to devise a medium which would allow rapid growth of strain ANU289 with minimum exopolysaccharide production. RGM30 is a defined minimal medium specifically designed to allow good growth of *Rhizobium* strain ANU289. Based on growth tests in media containing several carbon and nitrogen sources (Chapter 4), a new RGM30 derived medium containing gluconate (5g/l) and ammonium sulfate (1g/l) (called 'GAS' medium) was devised; this was used for selection of mutants. Ammonium was used as a nitrogen source rather than compounds such as glutamate, which have previously been used in *Rhizobium* media to avoid possible complications due to the nitrogen source also being used as a carbon source.
Table 5.4. Relative growth of the possible 36 mutants in liquid defined medium containing succinate or limited glucose and in complete medium

<table>
<thead>
<tr>
<th>isolates</th>
<th>succinate</th>
<th>limited glucose</th>
<th>complete medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU289</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>289-S-13</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>289-S-24</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-46</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-54</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-70</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-72</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-82</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-117</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-126</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-148</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-151</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-156</td>
<td>-</td>
<td>+</td>
<td>+++</td>
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<td>289-S-161</td>
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<td>+</td>
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<td>-</td>
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<td>289-S-228</td>
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<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-232</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>289-S-236</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>289-S-237</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>289-S-238</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>289-S-239</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>289-S-240</td>
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<td>+</td>
<td>+++</td>
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<td>-</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>289-S-244</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>289-S-247</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>289-S-265</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>289-S-269</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>289-S-277</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>289-S-281</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>289-S-282</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>289-S-284</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>289-S-286</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The isolates were grown in RGM30 based medium containing either succinate (20mM) or glucose (5mM) as carbon source and ammonium sulphate 10mM as nitrogen source. Complete medium contained 50mM glucose and 1% yeast extract. Growth was measured using Klett-Sommerson colorimeter after shaking incubation of 5-7 days.
A freshly-grown culture of strain ANU289 was mutagenised with 300 mg/l nitrosoguanidine for 6 hours and after washing in buffer the cell suspension was divided into two cultures. One was plated directly on GAS-Nm plates (GAS medium containing 300 mg/l neomycin). The other culture was grown for 24 hours in liquid GAS medium to allow phenotypic expression before being plated onto GAS-Nm. The relative growth of these mutants as scored visually is presented in Table 5.4.

Thirty six out of 99 isolates retained their inability to grow on succinate in liquid medium. Trace amounts of growth were observed by some mutant isolates, whereas a few others were found to be normal. This may be due to reversion, but was not followed up further as sufficient numbers of stable isolates were available. The growth on 5mM glucose (as sole carbon source) was also tested in these mutants. Most of the mutants which were unable to use succinate, were also unable to utilize limiting glucose efficiently. However, in complete medium, the growth of most of these isolates was normal. Some of the isolates, however, failed to grow in complete medium. The reason for this observation is totally unknown, and no further attempt was made to clarify this phenomenon. More importantly, the fact that 36 isolates were unable to grow on succinate alone indicated that either there was a defect in succinate uptake or metabolism. Some of them might have been uncoupled for oxidative phosphorylation since neomycin was used in the selection. These mutants, however, were not further characterized with regard to the nature of the lesions. Lack of growth or slow growth on limiting glucose of some Suc− mutants indicated a dependence on oxidative phosphorylation for growth.
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Figure 5.5. Selection procedure for succinate non-utilizing (Suc⁻) mutants. The neomycin resistant colonies were grown in complete medium containing neomycin (left) and medium containing succinate (20mM) as sole carbon source grown. The growth of wild type in the latter medium was more or less similar to that on complete medium (not shown). In the figure isolate 55 was chosen as a possible Suc⁻ mutant.
5.5.2 Nitrogenase activity in $\text{Suc}^-$ mutants and revertants

Since the major objective in isolating the $\text{Suc}^-$ mutants was to study the relationship between succinate metabolism and nitrogenase activity, it was essential to have some revertants. The mutants which were chosen for study of the above relationship were those characterised by the lack of growth on succinate, slow growth on limiting glucose and good growth on complete medium. When the test tube cultures in the preceding section (see Section 5.5.2) were allowed to incubate for longer periods, $\text{Sue}^+$ revertants of 5 $\text{NmR Sue}^-$ mutants were isolated. These appeared at a frequency of about $10^{-8}$, were neomycin sensitive, streptomycin resistant and capable of growth on succinate as was the parent strain ANU289. The nitrogenase activity of selected $\text{Sue}^-$ mutants (5 isolates) and of three of the revertants in stationary liquid culture is shown in Table 5.5. The table also shows the acetylene reduction of siratro nodules produced by these mutant strains and one of the revertant 289-S-13K.

The activity obtained in wild type strain ANU289 as a control was comparable to data previously obtained. With arabinose and succinate the wild type gave higher activity than in Chapter 4, since 50mM succinate was used in these experiment. The nitrogenase activity detected in mutant isolates varied but was always less than wild type in both arabinose and mannitol containing media. In the case of the arabinose containing medium the activities of the $\text{Sue}^-$ mutants were similar to that of the 'arabinose-only-control' for ANU289 in Chapter 4, indicating that succinate had no significant effect in $\text{Sue}^-$ mutants. This was also evident from the lack of change in pH after derepression had occurred, as it was shown that succinate utilization resulted in an alkaline shift of pH in the parent strain ANU289. The activities detected in the three
Table 5.5. Acetylene reduction in Suc\textsuperscript{−} mutants of strain ANU289 and their revertants

<table>
<thead>
<tr>
<th>Strains</th>
<th>Carbon Sources</th>
<th>Nitrogenase Activity (In planta)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate</td>
<td>Arabinose</td>
</tr>
<tr>
<td>ANU289 (Control)</td>
<td>427</td>
<td>653</td>
</tr>
<tr>
<td>Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>289-S-13</td>
<td>ND</td>
<td>35</td>
</tr>
<tr>
<td>289-S-24</td>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td>289-S-46</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>289-S-64</td>
<td>ND</td>
<td>13</td>
</tr>
<tr>
<td>289-S-148</td>
<td>7</td>
<td>39</td>
</tr>
<tr>
<td>Revertants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>289-S-13R</td>
<td>353</td>
<td>574</td>
</tr>
<tr>
<td>289-S-46R</td>
<td>392</td>
<td>384</td>
</tr>
<tr>
<td>289-S-148R</td>
<td>408</td>
<td>627</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Succinate (50mM) was used as supplementary carbon source in addition to mannitol and arabinose each at 50mM. Glutamate 3mM was used as nitrogen source. The activity was measured as nmols of ethylene per mg protein after 25 days. The readings are averages of five replicates.

In planta acetylene reduction was measured as µmoles/hr/g FW in siratro nodules. Reading are average of 6 plants, SE 0.7.
revertants were similar to that of the parent strain ANU289. In planta acetylene reduction data in siratro showed that all Suc\(^{-}\) mutants were defective in nitrogen fixation. Isolated bacteria from the sick-looking nodules of various mutants also exhibited neomycin resistance and inability to grow on succinate.

This indicated that succinate is one of the major carbon compounds responsible for the maintenance of nitrogenase activity in siratro nodules. This result is similar to those seen in *R. trifolii* (Ronson et al. 1981) and *R. leguminosarum* (Glenn and Brewin 1981) and lends weight to the assumption that even in *Bradyrhizobium* (Jordan 1982), succinate is a key metabolite in nodule function.

The conclusions that can be drawn from the above studies should be viewed in relation to the following points: (1) the mutants were obtained by nitrosoguanidine mutagenesis which causes multiple mutations (Shanmugam and Valentine 1980). However, the use of spontaneous revertants minimized this problem; (2) several other mutant isolates were tested for nitrogen-fixing nodulation in siratro (data not shown). Some mutants were unable to nodulate indicating perhaps there was a lesion in nodulation or 'fitness' gene(s); (3) the possibility of contaminants was ruled out by the colony phenotype on BMM plates and surveillance of genetic markers. Interestingly the majority of the Suc\(^{-}\) mutant isolates were able to nodulate siratro but were either unable to fix or fixed much less nitrogen. It was difficult, however, to conclude in the absence of revertants for all Nm\(^{R}\) Suc\(^{-}\) mutants, whether the ineffectiveness was due to the inability of cells to utilize succinate or simply due to neomycin resistance. It was reported previously that neomycin resistance usually led to ineffective symbiosis (Pankhurst 1977, Pariiskaya and Kalininskaya 1978, Rolfe and Gresshoff 1980, and Yakovelva 1981).
Additionally, neomycin resistant mutants were shown to be defective in heme synthesis in *E. coli* (Sasarman *et al.* 1975). Consistent with this, the ineffective mutants always produced a large number of small, white nodules in contrast to the few pink nodules formed by parent strain ANU289.

5.6 *Hydrogenase uptake mutants and their nitrogenase activity*

A relationship between hydrogenase activity and nitrogen fixation was first observed in *Azotobacter vinelandii* (Phelps and Wilson 1941), then in *Rhodospirillum rubrum* (Gest and Kamen 1949) where N₂ inhibition of photoevolution of H₂ led to the discovery of N₂ fixation in this and other photosynthetic bacteria (Kamen and Gest 1949). Evolution of H₂ from legume nodules was first reported by Hoch *et al.* (1957, 1960). More recently, it was established that H₂ was produced from the nitrogenase system and the loss of H₂ from nodules was suggested to be an 'unnecessary' expenditure of cellular energy, that may result in decreased efficiency of N₂ fixation (reviewed by Winter and Burris 1976). Because of the wide distribution of hydrogenase among a range of nitrogen-fixing microorganisms (Dixon 1978, Meyer *et al.* 1978, Walker and Yates 1978, Evans *et al.* 1980a, Evans *et al.* 1981, Bothe and Eisbrenner 1981) and since H₂ evolution by nodules of several legumes amounts to an average of about 30% of the total electron flow through the nitrogenase complex (Schubert and Evans, 1976, Evans *et al.* 1977, Carter *et al.* 1978, Ruiz-Argueso *et al.* 1978), the interest in H₂ metabolism of nitrogen-fixing organisms has recently increased.

Hydrogenase is an Fe-S protein that catalyses the oxidation and/or evolution of H₂ as shown below:

\[
\text{H}_2 + [\text{e}^- \text{carrier}] \text{(oxidised)} \rightarrow 2\text{H}^+ + [\text{e}^- \text{carrier}] \text{(reduced)}
\]
Hydrogenases may be uni- or bidirectional. *In vivo* the unidirectional hydrogenases (also called 'uptake hydrogenase'; Hup) mostly oxidise H\(_2\) in contrast to bidirectional hydrogenases, whose *in vivo* function depends on cell type and growth conditions (Arp and Burris 1981). Several functions proposed for these uptake hydrogenases in various legume nodules (Dixon 1972) were recently supported by the following experimental data. The oxidation of externally supplied H\(_2\) by H\(_2\)-uptake positive (Hup\(^+\)) bacteroids of soybean was shown to generate adenosine triphosphate (Ruiz-Argueso et al. 1979, Emerich et al. 1979), protect nitrogenase from oxygen damage (Emerich et al. 1979) and provide a mechanism for the conservation of carbon substrates (McCrae et al. 1978). Thus the uptake hydrogenases are enzymes whose *in vivo* characteristics appear to be adapted to H\(_2\) uptake rather than H\(_2\) evolution. They constitute a key portion of a H\(_2\) recycling process that may recover partly the energy that was expended by nitrogenase catalysed ATP dependent H\(_2\) evolution.

The distribution of strains tested for an effective uptake hydrogenase system in legume nodule is as follows: 19 R. *meliloti* (all Hup\(^-\)), 8 R. *trifolii* (all Hup\(^-\)), 61 R. *japonicum* (17% Hup\(^+\)), 13 cowpea Rhizobium (77% Hup\(^+\)) and 8 R. *phaseoli* (all Hup\(^-\)) (Lim 1978, Ruiz-Argueso et al. 1979, Evans et al. 1980a). A significant increase was obtained in yield, nitrogen content and N\(_2\)-fixation in soybeans and cowpeas inoculated with Rhizobium strains exhibiting Hup activity when compared with Hup\(^-\) strains (Schubert et al. 1978, Albrecht et al. 1979). A similar stimulatory effect was found when a Hup\(^+\) strain of R. *japonicum* was compared with a Hup\(^-\) mutant (Zablo towicz et al. 1980), but direct evidence for the beneficial effect was lacking, since the mutant did not revert and the lesions in other loci may have occurred during the selection process. Lepo et al. (1981) reported the isolation of
revertible Hup\textsuperscript{−} mutants of \textit{R. japonicum}. Plants inoculated with these mutant strains had lower dry weight and less total nitrogen than those inoculated with the parental Hup\textsuperscript{+} strain. Recently, Brewin \textit{et al.} (1980) demonstrated that the determinant for hydrogenase activity (Hup) in a \textit{R. leguminosarum} strain 128C53 was genetically linked to determinant for nodulation ability, both being carried on one plasmid.

Hydrogenase activity was expressed not only in \textit{R. japonicum} bacteroids but also in free-living cultures under defined conditions (Lim 1978, McCrae \textit{et al.} 1978, Maier \textit{et al.} 1979). The \(H_2\) uptake system in free-living \textit{R. japonicum} was characterised by several factors, namely induction by \(H_2\), repression by \(O_2\) and regulation by carbon substrates (Maier \textit{et al.} 1978\textit{a,b}, Ruiz-Argueso \textit{et al.} 1979, Lim and Shanmugam 1979). Moreover, Hup\textsuperscript{+} \textit{R. japonicum} strains grow chemolithotrophically, using \(H_2\) as an energy source (Hanus \textit{et al.} 1979, Lepo \textit{et al.} 1980).

Since the uptake hydrogenase is known to recycle \(H_2\) and thus provide energy to nitrogenase and strain ANU289 was moderately derepressable in terms of nitrogenase activity, the hydrogen metabolism in this strain was examined. The relationship between the uptake hydrogenase and nitrogenase, both in free-living cultures and in planta, was studied using Hup\textsuperscript{−} mutants of strain ANU289. These Hup\textsuperscript{−} mutants were produced using transposon Tn5 mutagenesis.

5.6.1 Uptake hydrogenase characterisation in strain ANU289

A technique employing triphenyl tetrazolium chloride (TTC) reduction (Maier \textit{et al.} 1978) was employed to examine the strains ANU288, ANU289, NGR231 and \textit{R. trifolii} strain Tl and \textit{R. japonicum} USDA122. Of these ANU288, ANU289, and USDA122 were Hup\textsuperscript{+}. This dye reduction technique was previously used as a screening procedure. The \textit{Rhizobium} strains having
hydrogenase readily reduced the dye (noted by a red colour) and were thus killed. For example, *R. trifolii* strain T1 was *Hup*- since no colour was produced on the TTC containing plate. Interestingly, the strain NGR231, another *Rhizobium* strain capable of nodulating *Parsponia* was *Hup*- in contrast to strains ANU288 and ANU289. The strain ANU289 was confirmed to be *Hup*+ by measuring hydrogen evolution from *Siratro* nodules using gas chromatography. No hydrogen was evolved from these nodules under a normal atmosphere of air.

5.6.2 Screening for *Hup*- mutants

Since strain ANU289 was shown to produce nodules containing the H₂-uptake system, it was necessary to isolate *Hup*- mutants to assess the role of hydrogenase in increasing the energy efficiency of nitrogen fixation in legumes and in free-living nitrogen-fixing cultures. The procedure used by most authors to select strains of *Rhizobium* defective in the uptake hydrogenase involved the determination of H₂ evolution by whole nodules and H₂ uptake by bacteroids. The production of nodules and bacteroids was cumbersome and time consuming, as screening of thousands of plants was required.

A two step method involving phenazine methosulfate (PMS) treatment followed by the triphenyltetrazolium chloride (TTC) procedure was devised by Haier et al. (1978) to select *R. japonicum* mutants unable to use hydrogen. The PMS effect was probably mutagenic. In *Azotobacter chroococcum* Postgate et al. (1982) followed a similar method for screening hydrogenase negative mutants. In these bacteria, slow reduction of methylene blue under H₂, after treatment with NaF and EDTA, distinguished *Hup*- from *Hup*+ colonies on plates. To isolate *Hup*- mutants in strain ANU289, a two step method such as transposon mutagenesis
followed by the TTC screening procedure was adopted. Tn5 mutagenesis has the additional advantage (see Rolfe et al. 1980) over NTG mutagenesis, used in the previous section, that only single mutational events occur. The sources for transposon Tn5 were E. coli strain 1830, JMP575, or ANU1041 (Chapter 2). To select for TTC negative phenotypes, the transconjugants were plated onto plates containing appropriate antibiotics (Kanamycin 600mg/l, Streptomycin 500 mg/l) to select for Tn5 and also 100mg/l of triphenyltetrazolium chloride (TTC). The transconjugants appeared on the plate 7–8 days after incubation; those which were hydrogenase positive reduced TTC and appeared red. The transconjugants lacking hydrogenase were unable to reduce TTC or only partially reduced TTC. These transconjugants were picked up as possible Hup<sup>−</sup> mutants and were tested for nodulation. Of 23 possible isolates, six were unable to take up hydrogen and were considered as Hup<sup>−</sup> mutants, namely 289-H-6, 289-H-9, 289-H-12, 289-H-14, 289-H-19 and 289-H-20.

5.6.3 Hydrogen evolution, hydrogen uptake and acetylene reduction by parent strain ANU289 and its Hup<sup>−</sup> mutants

Although the mutants isolated could be shown to be TTC negative, it was not known whether they were uptake hydrogenase negative. Therefore, these mutants were put into nodules of siratro and hydrogen evolution, hydrogen uptake and nitrogenase activity were measured (see Table 5.6). The parent strain (ANU289) showed no hydrogen evolution in air. The mutants showed variable amounts of hydrogen evolution. Similarly, only strain ANU289 exhibited uptake hydrogenase activity when hydrogen was exogenously supplied and its disappearance measured. The differences between the parent strain and the mutant were clear cut. A difference was noted in the nitrogenase activity (measured by acetylene reduction) of
Table 5.6. Hydrogen evolution and hydrogen uptake by parent strain ANU289 and its Hup\textsuperscript{−} mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hydrogen Evolution (umoles/hr/g FW)</th>
<th>Hydrogen Uptake (umoles/hr/g FW)</th>
<th>Acetylene Reduction (umoles/hr/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU289</td>
<td>0.001</td>
<td>1.2</td>
<td>9.5 ± 0.7</td>
</tr>
<tr>
<td>289-H-6</td>
<td>7.8 ± 1.3</td>
<td>0.001</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>289-H-9</td>
<td>5.6 ± 0.5</td>
<td>0.001</td>
<td>3.6 ± 1.2</td>
</tr>
<tr>
<td>289-H-12</td>
<td>7.3 ± 2.5</td>
<td>0.001</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>289-H-14</td>
<td>6.3 ± 3.2</td>
<td>0.001</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>289-H-19</td>
<td>5.5 ± 1.7</td>
<td>0.001</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>289-H-20</td>
<td>3.5 ± 0.7</td>
<td>0.001</td>
<td>6.0 ± 0.7</td>
</tr>
</tbody>
</table>

The readings are averages of 6 siratro plants. The nodule number on plants varied from 3 to 8. The hydrogen evolution or uptake was measured by gas chromatography. The vials containing nodules were injected with 1% hydrogen and the disappearance (uptake) was measured at 1h intervals.
these parent strain- and mutant isolates-induced nodules. The parent strain ANU289 showed the highest nitrogenase activity. The activity was reduced in all the mutants but to different degrees, giving an average reduction of in planta activity of about 35%. The hydrogen evolution and uptake results confirm the fact that the mutants under examination are Hup⁻ mutants.

The mechanism of TTC action is not known. This chemical was used in screening procedures because Hup⁻ strains did not reduce the dye whereas Hup⁺ strains reduced the dye resulting in pink colonies (Maier et al. 1978). In fast-growing R. trifolii, reported to lack uptake hydrogenase (Ruiz-Argueso et al. 1979), TTC was used to select succinate transport mutants (Ronson et al. 1981). In the presence of succinate, these bacteria produced alkali due to their symport with protons as in the case of E. coli (Gutowski and Rosenberg 1975). Slow-growing rhizobia (e.g. 'cowpea strains' or R. japonicum) are also similar to R. trifolii in this regard. However, Hup⁻ mutants were not selected with succinate containing medium here. Since hydrogenase is a membrane-bound protein (Pinkwart et al. 1979) it is possible that in Hup⁺ strains the protons are oxidised to H₂ by hydrogenase thereby promoting alkaline conditions outside the cell and reducing the dye. Observations that strain Tl on the same medium did not reduce the dye indicated that it was not a succinate-like effect. The decreased acetylene reduction observed in Hup⁻ mutants compared to the parent strain may have been because of their inability to recycle some of the energy. These results are in agreement with Maier et al. (1978).
5.6.4 Nitrogenase activity in free-living culture of \textit{Hup}^- mutants

One of the major objectives in isolating \textit{Hup}^- mutants was to determine whether the \textit{Hup}^+ phenotype conferred benefits on the culture with respect to the nitrogenase expression. Work of this type has not been reported in the literature. Table 5.7 shows the effect of the presence of uptake hydrogenase on nitrogenase activity in culture.

After 7 days the parent strain ANU289 showed twice as much activity as the mutants. The activity of strain ANU289 was comparable to previous findings with arabinose (50mM) and succinate (50mM) as carbon sources (Chapter 4). The revertants isolated from two of the Tn5 mutants, 289-d-6R (the revertant became Ka sensitive) and 289-H-9R (the revertant was still Km resistant indicating the Tn5 had transposed) showed activity comparable to that of wild type. Since Tn5 induces random single mutagenic events (see Rolfe et al. 1980), it is likely that the decline in nitrogenase activity in these mutants compared to the \textit{Hup}^+ parent strain and \textit{Hup}^+ revertants may be due to a lack of uptake hydrogenase activity (\textit{Hup}^-).

Also shown in Table 5.8 is the effect of additions of 1\% hydrogen to these derepressed cells after 7 days. When the nitrogenase activity was measured at 11 days, the presence of 1\% hydrogen in the gas phase appeared to stimulate nitrogenase activity in the parent strain and revertants (all \textit{Hup}^+) but not in the \textit{Hup}^- mutants. Since succinate played a critical role in derepression of nitrogenase activity and these cultures were derepressed with 50mM succinate, it was possible that the stimulatory effect was due to the interaction of both succinate and hydrogen metabolism. Such stimulatory effect of hydrogen on succinate derepressed bacteroids of \textit{R. japonicum} was previously reported (Emerich et al. 1979).
Table 5.7. Nitrogenase activity of strain ANU289 and its Hup⁻ mutants

<table>
<thead>
<tr>
<th>isolates</th>
<th>activity after 8 days</th>
<th>activity after 11 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without H₂</td>
<td>with H₂ (1% v/v)</td>
</tr>
<tr>
<td>ANU 289</td>
<td>766</td>
<td>839</td>
</tr>
<tr>
<td>289-H-6</td>
<td>308</td>
<td>443</td>
</tr>
<tr>
<td>289-H-9</td>
<td>370</td>
<td>488</td>
</tr>
<tr>
<td>289-H-12</td>
<td>202</td>
<td>339</td>
</tr>
<tr>
<td>289-H-14</td>
<td>279</td>
<td>377</td>
</tr>
<tr>
<td>289-H-19</td>
<td>199</td>
<td>597</td>
</tr>
<tr>
<td>289-H-20</td>
<td>203</td>
<td>292</td>
</tr>
<tr>
<td>289-H-6R</td>
<td>690</td>
<td>734</td>
</tr>
<tr>
<td>289-H-9R</td>
<td>824</td>
<td>836</td>
</tr>
</tbody>
</table>

The derepression medium contain 50mM arabinose and 50mM succinate as carbon sources and 3mM glutamate as nitrogen source. Values are average of 4 replicates. The activity was measured as nmoles ethylene per mg protein after 8 and 11 days. Pure hydrogen gas was injected into the half of the vials after 8 days, so that the gas phase contained 1% hydrogen.
5.7 Summary

To understand the regulation of nitrogenase activity in strain ANU289, the physiological studies of Chapter 3 and 4 were further extended using genetic analysis. Three major, probably interrelated aspects involved in energy metabolism which presumably controlled the level of nitrogenase activity both in vitro and in planta were studied. This involved isolating mutants distinctly defective either in EPS synthesis (Muc⁺), succinate metabolism (Suc⁻) or hydrogen uptake (Hup⁻) and subsequent analysis of the effects of these mutations on nitrogenase activity.

The investigations dealing with the relationship between EPS synthesis and nitrogenase activity clearly delineated a negative correlation between these two parameters. This was evident from both EPS defective (Muc⁺) mutant analysis on mannitol containing media and was later confirmed using the isogenic strains ANU288 (mucoid) and ANU289 (non-mucoid) with six other carbon sources. Keeping the genetic system constant (using only strain ANU289), when 15 different carbon sources were used to study EPS synthesis and nitrogenase activity, an inverse relationship was also apparent. These studies indicated an overall complex relationship among several metabolic processes such as oxygen consumption, growth (protein synthesis), EPS production, nitrogenase activity and probably some other unknown factors. A simplified view of this 'black box' is diagrammatically presented as an energy flow scheme (Fig. 9.1).

Though several reports are documented in the literature regarding isolation of succinate transport mutants, all of them dealt with fast-growing rhizobia. This is perhaps the first report of succinate metabolism defective mutants in slow-growing Rhizobium species. The
mutants of strain ANU289 defective in either succinate uptake or metabolism demonstrated a definite positive role of succinate in derepression of nitrogenase activity in both free-living micro-aerobic conditions and legume nodules. Inability to utilize succinate drastically reduced both \textit{in vitro} and \textit{in planta} nitrogenase activity. However, succinate was not the only source of energy and reducing equivalents in free-living cultures since activity was detected with other carbon sources.

Similar to the Suc\textsuperscript{−} mutants, analysis of Hup\textsuperscript{−} mutants demonstrated a role of the uptake hydrogenase not only in nitrogen fixation of siratro nodules but more importantly in \textit{in vitro} cultures. The experiments established that both strains ANU288 and ANU289 are uptake hydrogenase positive (Hup\textsuperscript{+}). Reduced nitrogen fixation in siratro nodules produced by Hup\textsuperscript{−} mutants of strain ANU289 suggested an involvement of Hup in nitrogen fixation efficiency probably via an improved 'energy household' of the cell. Effects of Hup similar to those in nodules were observed for the first time in \textit{in vitro} conditions.
CHAPTER-6

CONTROL OF NITROGENASE ACTIVITY

BY NITROGENOUS COMPOUNDS
6. CONTROL OF NITROGENASE ACTIVITY BY NITROGENOUS COMPOUNDS

6.1 Nitrogen control and ammonium assimilation in Klebsiella

*Klebsiella pneumoniae*, a member of *Enterobacteriaceae* is undoubtedly the most researched among identified diazotrophs for what is known as 'nitrogen control'. This phenomenon refers to the co-ordinate control of several nitrogen assimilatory enzymes (e.g. proline utilization, *put*; histidine utilization, *hut*, nitrogen fixation, *nif*, etc.) by the intracellular concentration of NH$_4^+$. Initially studies on the regulation of histidase synthesis in *Klebsiella aerogenes* led Magasanik and co-workers (Magasanik et al. 1974) to formulate a model in which the principal regulator of nitrogen assimilation was the enzyme glutamine synthetase (GS). It was postulated that GS was responsible for the biosynthesis of glutamine as well as for the activation of transcription of other operons including those of nitrogen fixation genes (*nif*). GS activity was regulated by the reversible adenylylation of the specific tyrosyl residues on each subunit of this dodecameric protein. Thus it was proposed that in conditions of nitrogen starvation, GS was deadenylylated and highly active and was responsible for initiating transcription of the *nif* operons (see review by Magasanik 1982). The last three years have seen considerable work leading to the emergence of a very different model for regulation of nitrogen assimilation. Thus GS is no longer considered to be a regulatory protein, instead products of three genes *glnF*, *glnG* and *glnL* are sufficient to account for the regulation of these assimilatory enzymes. Although the activity of deadenylylated GS may control the nitrogen status of cells, it plays no role in activating the other assimilatory operons.

A revised model for regulation of these nitrogen assimilatory operons, particularly those of the *nif* operons is presented in Fig. 5.1 (Ow and Ausubel 1983). The central 'nitrogen control (see figure legend)' operates via two regulatory genes, namely *nifL* and *nifA*, located in a single operon within the *nif* cluster. Of these, the *nifL* gene product acts in the presence of NH$_4^+$ and oxygen to repress transcription of the other *nif* operons (Hill et al. 1981, Buchanan-Wollaston et al. 1981).
Conversely, _nifA_ autoactivates its own promoter, as well as other _nif_ promoters and the _glnA_ promoter under nitrogen-limited and low oxygen conditions (Ow and Ausubel 1983).

Depending on the relative availability of ammonium and energy, ammonium assimilation occurs in _Klebsiella_ in either of the following two ways: When ammonium is present in excess relative to the energy sources, GS is repressed and glutamate dehydrogenase (GDH), which reductively aminates α2-ketoglutarate to yield glutamate (see reaction 1) is derepressed. However, with limited ammonium, GS and glutamate synthase (GOGAT, L-glutamine: 2-oxoglutarate aminotransferase) operate in concert to yield the same net result (see reactions 2 and 3) with the simultaneous hydrolysis of 1 mole of ATP per mole of net glutamate formed (Meers et al. 1970). These reactions are as follows:

\[
\begin{align*}
2\text{-}\text{keto} \text{glutarate} + \text{NH}_4^+ & \rightarrow \text{L-glutamate} \quad \text{GDH} \\
& \quad \text{NADPH} \\
\text{L-glutamate} + \text{NH}_4^+ + \text{ATP} & \rightarrow \text{L-glutamine} + \text{ADP} + \text{P}_i \quad \text{GS} \\
\text{L-glutamine} + 2\text{-keto} \text{glutarate} & \rightarrow 2\text{L-glutamate} \quad \text{GOGAT} \\
& \quad \text{NADPH}
\end{align*}
\]

6.1.1 Pathways of ammonium assimilation in _Rhizobium_ and GS regulation of nitrogenase activity

In contrast to _Klebsiella_, ammonium assimilation in _Rhizobium_ species is more complicated. GDH was reported to be present in _R. meliloti_ strain 41 (Kondorosi et al. 1977), strain 2011 (Osbourne and Signer 1980, Ali et al. 1981), _R. leguminosarum_, _R. trifolii_ and _R. japonicum_ (Brown and Dilworth 1975). It was suggested that this enzyme (GDH) may play a role in ammonium assimilation.
However, in some other Rhizobium strains such as 32H1 (Rhizobium sp.) and 6A176 (R. japonicum), which derepress nitrogenase in vitro, GDH was absent and GS-GOGAT constituted the sole pathway of ammonium assimilation (Ludwig 1978, 1980). In addition most of the Rhizobium strains were found to possess two GSs, GSI and GSII (Darrow and Knotts 1977, Fuchs and Keister 1980a, 1980b, Ludwig 1980a, Darrow et al. 1981, Howitt 1982). In accordance with the earlier model of GS regulation of nitrogen control (See Section 6.1) in Klebsiella, it was possible that GS might be involved in the regulation of nitrogenase in rhizobia. However, evidence both in favour and against the involvement of GS in nitrogenase regulation has been reported.

The addition of ammonium to microaerobic and nitrogen-fixing cultures inhibited nitrogenase activity but the level of GS and its adenylylation properties remained unaltered (Scowcroft et al. 1976, Darrow and Knotts 1977, Rao et al. 1978). These studies provided little support for a role of GS in nitrogenase regulation. In contrast, others have found a positive correlation between decreased adenylylation and nitrogenase activity in O₂-limited continuous cultures (Bergersen and Turner 1976). If O₂-limitation was partially relieved, nitrogenase activity was inhibited by ammonium, simultaneously there was an increased adenylylation of GS. Furthermore, of the two GSs in strain 32H1, GSI appeared to undergo adenylylation and be involved in the control of nif expression (Ludwig 1980b). GSII, which was also repressed by ammonium, was suggested to function in purine biosynthesis but not in ammonium assimilation.

Mutants with low GS activity were isolated in strain 32H1 (Ludwig and Signer 1977) and R. meliloti strain 41 (Kondorosi et al. 1977) and these lacked nitrogenase activity. Particularly in strain 32H1, the nitrogen fixation defects were shown to be the direct result of glutamine
auxotrophy as reversion to prototrophy simultaneously recovered nitrogenase derepression ability. Thus, a GS mediated nitrogenase expression was postulated consistent with early models of nitrogen control in Klebsiella (Section 6.1) (Ludwig 1981). Whether this phenomenon is analogous to a glnG mutation in Klebsiella remains unanswered in the absence of physical data for gln genes in rhizobia.

As nifA and glnG have substantial homology (Ow and Ausubel 1983) and nifA of Klebsiella activates transcription of nif structural genes of R. meliloti (Sundareson 1983), a common control mechanism has been envisaged among these nitrogen fixing bacteria. Very recently glnA gene from R. japonicum strain 110 (Carlson et al. 1983) and GSI gene from R. meliloti (Kahn and Somerville 1983) have been cloned. Further analysis of other gln genes and nif regulatory genes is necessary before any comments on nitrogen regulation in rhizobia can be made.

Compared to GSs, GOGAT has received less attention. GOGAT defective mutants were isolated (Kondorosi et al. 1977, Osburne and Signer 1980). These were able to nodulate and fix nitrogen normally indicating that GOGAT may not have a significant role in the regulation of nitrogen fixation.

6.1.2 Ammonium effects on nitrogenase activity in Rhizobium

In asymbiotic free-living diazotrophs (e.g. Klebsiella) ammonium inhibited nitrogenase activity both by repression of nitrogenase synthesis as well as by modulation of activity (see Eady 1981). In Rhizobium, conflicting views have been expressed in the literature regarding the effects of ammonium on nitrogenase. For example, 10mM ammonium did not inhibit nitrogenase activity in agar slopes of strain 32H1 (Gibson et al. 1976) or 61A76 (Kurz and La Rue 1975) and also in shaken liquid cultures of these strains (Keister and Evans 1976). However, at the same
concentration of ammonium, nitrogenase activity was inhibited in stationary liquid cultures of 32H1 and 311b83 (Evans and Keister 1976). Inhibition occurred even at a lower concentration (5mM) in disc cultures with 0.2 atm O₂ (Scowcroft et al. 1976), but in this study, ammonium inhibition was relieved by increased oxygen tension. Thus, the inconsistency of ammonium effects in these reports was probably due to inadequate methodology in which factors such as O₂ concentration, pH or carbon sources were not appropriate.

Using optimal conditions for nitrogenase derepression (i.e. low oxygen, glutamate as N-source), the addition of 10mM NH₄Cl to the already derepressed cultures caused a loss in activity of about 70-80% in R. japonicum strain 61A76 and somewhat less with strain USDA83 and 30-35% with Rhizobium sp. 32H1 (Keister and Ranga Rao 1976, Rao et al. 1982). The inhibitory effect of ammonium varied not only with the strains tested but also with the ammonium salt used. For glutamine limited continuous cultures of strain 32H1, the addition of 5mM NH₄⁺ reduced the nitrogenase activity by 50% with an apparent half life of 90 min (Bergersen et al. 1976). In oxygen-limited cultures of cowpea strain CB756, an increase of added ammonia from 1.5mM to 3.4mM reduced nitrogenase by only about 20-30% (Bergersen et al. 1976). During a 90 min incubation period, no such effect after the addition of 10 and 100mM ammonium was observed by Werner and Stripf (1978) in R. japonicum strain 61A101.

Parallel to these results with cultured rhizobia, data from isolated R. leguminosarum bacteroids showed that ammonium (10mM) did not influence nitrogenase activity (Laane et al. 1980, Houwaard 1979, 1980). Furthermore, incubation of isolated bacteroids in 10 or 25mM NH₄⁺, 10mM NO₃ or 10mM NO₂ did not specifically influence nitrogenase
synthesis (K.C. Van den Bos pers. comm.). Thus in general, nitrogenase activity in rhizobia was relatively less sensitive to ammonium inhibition compared to other free-living 'nitrogen-fixers'. This inhibition in rhizobia was more likely due to modulation of activity rather than repression of synthesis.

Several nitrogen sources were previously tested for their ability to support growth and the derepression of nitrogenase activity in strain ANU289 (see Section 4.4). Though the majority of these derepressed nitrogenase activities, optimal activity was only observed with glutamate, proline and casamino acids. Ammonium and glutamine, both of which are by-products of nitrogen assimilation inhibited nitrogenase activity. This chapter, therefore, focuses on the effects of these compounds on nitrogenase activity with the aim of further clarifying the similarities and dissimilarities of strain ANU289 with respect to nitrogen metabolism and nitrogenase derepression of other Rhizobium strains or free-living 'nitrogen-fixers'. To this end attempts to isolate ANU289 mutants defective in glutamine assimilation, as well as derepression studies with these are also described.

6.2 Methods

The derepression techniques are described in Section 2.3.2., as are the assay methods for glutaminase and glutamine uptake systems in Section 2.3.4. The procedures of isolating mutants defective in ammonium assimilation have also been described (see Section 2.3.3).
6.3 Growth kinetics of strain ANU289 in several nitrogenous compounds

In previous chapters, studies with different carbon sources showed that individual compounds substantially influenced both growth and nitrogenase activity. Although extensive studies of this nature have not been done in other rhizobia with respect to nitrogenous compounds, from the relative growth measurements (see Table 4.4) a definite relationship was not apparent. Also, because of the tight co-ordination between carbon and nitrogen metabolism, the available carbon sources may influence growth on different nitrogen sources. A quantitative growth study was therefore undertaken. The growth kinetics of each of these compounds with glucose or arabinose as carbon source is depicted in Fig 6.2A and 6.2B.

Irrespective of the sugar used, no growth was observed in the absence of nitrogen supplied nor if methylamine was used as a nitrogen source. Nitrate, ammonium sulphate, acetamide and glutamate supported similar growth kinetics on either sugars. Acetamide and glutamate supported maximal growth, whereas intermediate and poor growth was seen on NO₃ and ammonium, respectively. Urea supported vigorous growth with arabinose but not glucose. Casamino acids showed intermediate growth on arabinose and poor growth with glucose. In contrast, histidine exhibited better growth in combination with glucose rather than with arabinose.

Thus all nitrogenous compounds tested supported growth except methylamine. The absence of growth in methylamine may be due to the absence of an uptake system, as is the case with strain 32H1 under aerobic conditions (Gober and Kashket 1983). Interestingly acetamide has been very seldom tested as a carbon or nitrogen source in *Rhizobium*, but it is a good candidate because of its utilization by *Pseudomonas* (Kelly and Clarke 1962). Growth on these nitrogen sources suggest that the enzyme systems for their assimilation exist in strain ANU289. The presence of
Figure 6.1. Growth kinetics of strain ANU289 using a range of nitrogen sources namely (1) KNO$_3$, (2) NH$_4$Cl, (3) acetamide, (4) urea, (5) methylamine, (6) L-histidine, (7) glutamate and (8) casamino acids. Except casamino acids (0.1%) all other nitrogen sources were present at 10mM N. Glucose (at 50mM) served as carbon source. The medium was buffered with MOPS (pH 6.8).
The diagram shows the growth of various strains labeled 1 to 8 over 5 days in klett units. The x-axis represents days, ranging from 0 to 5, and the y-axis represents glucose levels, ranging from 0 to 500. Each strain shows a unique growth pattern over the 5 days.
Figure 6.2. Growth kinetics of strain ANU289 on a range of nitrogenous compounds as described in Figure 6.1. Arabinose (50mM) served as carbon source. The medium was buffered with MOPS (pH 6.8.).
The enzyme systems have been recently demonstrated by E. coli (Park, 1970). An interaction between carbon and nitrogen sources was observed from the differential utilization of certain carbon sources such as arabinose or histidine. Lack has not been studied, although lack of arabinose caused normal growth. It showed optimal nitrogen depression. There was no relationship between the ability of a compound to support growth and stimulate activity as is seen in certain components (e.g., glutamine) that nitrogen compounds normally support. However, the effect of glutamine on arabinose assimilation was not observed in the nitrogen compounds tested. Glutamine supported optimal activity of this enzyme was the inhibition of a certain arabinose assimilation. The logarithmic growth of one of the nitrogen compounds was found to be 0.1, 0.5, 1.0, 1.5, and 2.0 klett units after 0 days in strain AB6279. By comparison, the uninhibited nitrogen activity was inhibited by 1.0 in strain AB6279. In strain 1011, out glutamate under similar conditions, activity was inhibited by 1.0 in strain AB6279. In strain 1011, out glutamate under similar conditions, activity was inhibited by 1.0 in strain AB6279. In strain 1011, out glutamate under similar conditions, activity was inhibited by 1.0 in strain AB6279.
these enzyme systems have been recently demonstrated by S. Howitt (pers. comm.). An interaction between carbon and nitrogen sources was indicated from the differential utilization of certain nitrogen compounds such as urea or histidine. Last but not least, although glutamate supported maximal growth, it showed optimal nitrogenase derepression. Thus there was no relationship between the ability of a compound to support growth and stimulate activity as is seen in carbon compounds (see Chapter 5). Nitrogen compounds probably influence nitrogenase derepression by some other mechanism other than supporting growth itself. Because of the interaction between carbon and nitrogen sources under both aerobic and microaerobic conditions (not shown), it appears that nitrogenous compounds may act by signalling for N-starvation caused by the carbon:nitrogen ratio of the cell.

6.3.1 Effect of glutamine on nitrogenase activity

Of the nitrogenous compounds tested, only glutamate, proline and casamino acids supported optimal activity in strain ANU289. Of particular significance was the inhibition of activity by glutamine, the first incorporation product of ammonium assimilation. Glutamine was previously found to be the best nitrogen source for supporting nitrogenase activity in agar slopes of strain 32H1 (Gibson et al. 1976, Pankhurst 1981), and with strain CB756 in continuous cultures (Bergersen et al. 1976). Due to this contrast with closely related 'cowpea strains', the effect of glutamine on nitrogenase activity in strains ANU289, CB756 and 32H1 was compared using stationary liquid culture conditions (Table 6.1).

Nitrogenase activity was derepressed with different levels of glutamine such as 0, 1, 3, 5, 10 and 20mM, in addition to pre-existing 3mM glutamate already present in the medium. The addition of 1mM glutamine as extra nitrogen to the medium inhibited acetylene reduction by about 90% after 16 days in strain ANU289. By comparison, at this concentration, nitrogenase activity was inhibited by 33% in strain CB756. In strain 32H1, 5mM glutamine caused only 50% reduction in the total amount of
Table 6.1. Comparison of acetylene reduction in selected *Rhizobium* strains at different initial glutamine levels

<table>
<thead>
<tr>
<th>Initial glutamine levels (mM)</th>
<th>Acetylene reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANU289</td>
</tr>
<tr>
<td>0</td>
<td>911 (100)</td>
</tr>
<tr>
<td>1</td>
<td>92 (10)</td>
</tr>
<tr>
<td>5</td>
<td>54 (6)</td>
</tr>
<tr>
<td>10</td>
<td>18 (2)</td>
</tr>
<tr>
<td>20</td>
<td>16 (2)</td>
</tr>
<tr>
<td>40</td>
<td>15 (2)</td>
</tr>
</tbody>
</table>

Arabinose (50mM) and succinate (50mM) were used as carbon sources. Glutamate (3mM) served as nitrogen source. Data represent acetylene reduction in nmol of ethylene per mg protein after 16 days, and are averages of at least five replicates. Values in parentheses represent standardised percentages. Initial cell density was 20% of a RGM30M grown late-logarithmic phase culture. Initial oxygen levels were at 10%.
acetylene reduced, and an increase up to 20mM caused only about 70% inhibition. The control activity, with glutamate alone, was higher than previously observed, due to more optimised conditions (such as 50mM succinate) used in this experiment.

Thus nitrogenase activity in other 'cowpea strains' such as 32H1 and CB756 was definitely less sensitive to glutamine inhibition than was found for strain ANU289. Whether this phenomenon is caused by decreased uptake of glutamine in other cowpea strains or altered pathways of ammonium assimilation in strain ANU289 is, as yet, unclear. Glutamine, if readily taken up, may influence the α-ketoglutarate:glutamine ratio, thus signalling nitrogen abundance and leading to the repression of all nitrogen assimilatory enzymes, including nitrogenase.

6.3.2 Effect of added glutamate, glutamine and ammonium on derepressed nitrogenase

Interactions between amino acids and nitrogenase expression were reported in R. japonicum strain 61A76 (Rao et al. 1982). When glutamine, asparagine, leucine, phenylalanine, urea, casamino acids or yeast extract were added to glutamate containing medium nitrogenase activity was inhibited. Yeast extract (0.1%) showed maximum inhibition (up to 90%) followed by leucine, glutamine, asparagine (each at 10mM) and casamino acids (0.1%). In section 6.3.2 it was shown that nitrogenase activity of strain ANU289 was more sensitive to glutamine than strains CB756 and 32H1. It was not known whether the effect was solely due to glutamine or the result of its interaction with glutamate (3mM) also present in the derepression medium. It was therefore necessary to derepress activity with some nitrogen source other than glutamate (e.g. casamino acids) and then to examine the effect of the addition of other nitrogen compound of interest. The effect of ammonium, glutamine or glutamate on the activity using this technique is shown in Table 6.3.2.
Table 6.2. Effect of added glutamine, glutamate and ammonium sulphate on acetylene reduction in strain ANU289.

<table>
<thead>
<tr>
<th>Nitrogen sources</th>
<th>Concentration (1mM N)</th>
<th>Concentration (5mM N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before 2 days %</td>
<td>before 2 days %</td>
</tr>
<tr>
<td></td>
<td>after increase</td>
<td>after increase</td>
</tr>
<tr>
<td>Control</td>
<td>128 306 100</td>
<td>128 306 100</td>
</tr>
<tr>
<td>glutamate</td>
<td>130 340 99</td>
<td>122 296 92</td>
</tr>
<tr>
<td>glutamine</td>
<td>140 324 82</td>
<td>129 159 53</td>
</tr>
<tr>
<td>ammonium sulphate</td>
<td>135 308 77</td>
<td>156 163 9</td>
</tr>
</tbody>
</table>

Acetylene reduction was measured after 4 days of incubation in medium containing arabinose (50mM), succinate (20mM) and casamino acids (0.05%) with 5% oxygen in gas phase; once more by 6 days.

Sterilized glutamate, glutamine and ammonium sulphate was added at appropriate concentration and activity then measured expressed as nmoles of ethylene/vial (27 ml), readings are average of 10 replicates.
Nitrogenase activity was measured after 4 days of incubation and then the indicated nitrogen compounds were injected aseptically to produce the appropriate concentration. Activity was measured after 2 more days and the effects were standardised relative to a control, which received H₂O as an addition. Glutamate had a slightly negative effect at 5mM and no effect at 1mM. Both glutamine and ammonium sulphate caused a decline of nitrogenase activity by 20-25% at 1mM. At 5mM, the inhibition was more significant as glutamine reduced the activity by 50% and ammonium by 90%. The glutamine effect was not as severe in this case as was previously found with glutamate derepressed cultures (Section 6.3.2). Thus, the interaction with casamino acids appears to alter slightly the inhibitory response to glutamine. These experiments suggested an inhibitory effect of both ammonium and glutamine and are thus in agreement with Evans and Keister (1976), Keister and Ranga Rao (1976), Rao et al (1982), and Bergersen et al. (1976). Further, while it is not known whether ammonium or glutamine repress nitrogenase synthesis, this experiment demonstrated that they certainly have an inhibitory effect on activity.

6.3.3 Ammonium inhibition in rapidly agitated liquid cultures

Although an inhibitory effect of ammonia was consistently observed, it may have been a consequence of the methodology being used, i.e. stationary liquid culture (see Section 6.3.2). In several strains this inhibition of nitrogenase by ammonium disappeared when shaken liquid cultures were used with controlled low oxygen concentrations in the gas phase (Keister and Evans 1976, Rao et al. 1982).

To resolve this, ammonium inhibition was further studied in rapidly agitated liquid cultures. The cultures were derepressed with 50mM succinate (as carbon source) and 3mM glutamate (as nitrogen source),
Figure 6.3. Ammonium inhibition of established nitrogenase activity in rapidly agitated cultures of Rhizobium strain ANU289. Succinate (50mM) and glutamate (5mM) were supplied in the medium as carbon and nitrogen sources. Oxygen was maintained in the range of 0.2 - 0.25% in the gas phase. Cultures in duplicate were agitated at 170 rpm at 28°C. Ammonium chloride or MSX (filter-sterilised) was added after 40 h of incubation. Time 0 on figure represents the point of injection of the tested compounds. Control was injected with sterile H₂O.
Figure: The effect of ammonium (NH$_4^+$) on nitrogenase activity. The activity was reduced by addition of ammonium, and the rate of activity remained constant for the control for 15 hours after injection. The addition of ammonium to the uninhibited strain also caused a decline in activity, although the inhibition of control strain would prevent the respiration of any glutamine. The addition of ammonium to the uninhibited strain (MSX at 120) caused a further decline in activity, but there was no further decline in activity for 15 hours after injection.
and 5mM NH₄Cl was added after 60 hours of incubation. Oxygen levels during these experiments were kept near constant in the range of 0.2-0.25%. The data are presented in Figure 6.3.

The addition of ammonium (at 5mM) rapidly inhibited the observed acetylene reduction. The activity was reduced by 50% compared to that of the control 5 hours after injection. The rate of activity remained constant for the control for about 8 hours after injection. Further elucidation of the inhibition by ammonium was carried out by using the inhibitor methionine sulfoximine (MSX). MSX is a transition state inhibitor of GS, and therefore has a very high affinity for GS. Inhibition of GS by MSX would prevent the formation of any glutamine, so the level of glutamine inside the cell should fall. The addition of methionine sulfoximine (MSX at 10mM) to the derepressed cultures had no significant effect (other than perhaps a slight increase) on the control rates. However, MSX (at 10mM) completely removed ammonium inhibition. MSX plus NH₄Cl actually resulted in a noticeable increase in the rate of acetylene reduction. Similar effects with NH₄Cl were seen at 2mM, although quantitatively the inhibition of nitrogenase was less pronounced (not shown).

Thus, in addition to being sensitive to glutamine and NH₄⁺ in stationary cultures, strain ANU289 was also shown to be more sensitive to ammonium in rapidly agitated cultures. Hence the inhibition of nitrogenase activity by ammonium and glutamine observed here is not an artifact but probably a characteristic of this strain in contrast to other cowpea rhizobia such as 32H1 and CB756.
6.4. Glutamine uptake by cultured cells of strain ANU289

Whilst both ammonium and glutamine inhibited nitrogenase activity in strain ANU289 and the inhibition by the former was relieved by MSX indicating intracellular glutamine levels may be involved in the control of nitrogenase expression, whether exogenous glutamine inhibition of nitrogenase activity was due to its effect on endogenous levels remained unclear. No report of glutamine uptake in Rhizobium exists in the literature, therefore glutamine uptake was studied in strain ANU289.

Figure 6.4 shows the uptake of glutamine by this strain with cells grown on either glutamate and glutamine as the sole nitrogen source. Rapidly agitated cells were exposed to a range of glutamine concentrations containing $^{14}$C glutamine over a period of 17 min. The uptake of $^{14}$C glutamine was consistently 30% greater by glutamine grown cells than by glutamate grown cells, over the concentrations ranging from 0.1mM to 3mM. At 10 and 30mM glutamine there was about a 50% increase in uptake of labelled glutamine by cells grown on glutamine. Glutamine uptake thus appeared to be substantially constitutive. Increased uptake by glutamine grown cells indicated that the uptake was also partly inducible. Interference of glutamine uptake in the presence of external glutamate suggested that there existed an interaction between these compounds even at the uptake level.

6.4.1 Glutaminase activity in culture cells

The presence of a glutamine-uptake system even in glutamate grown cells of strain ANU289 was significant in view of glutamine effects on nitrogenase activity. The fate of glutamine inside the cell remained unknown. It was necessary to study whether GOGAT was the only enzyme assimilating glutamine or whether other enzymes existed. In Escherichia coli under unfavourable conditions, another enzyme 'glutaminase
Figure 6.4. Glutamine uptake by strain ANU289 pre-grown in either glutamate or glutamine. The uptake of $^{14}$C glutamine was measured as nmoles glutamine mg$^{-1}$ protein min$^{-1}$ (see the text for details).
Glutaminase was also reported to duplicate glutamate dehydrogenase (EC 5.4.1.2) in the assay switched off the major biosynthetic pathways and maintained energy. Glutaminase was also reported in the literature of by saturating and glutamic acid, which has a strong negative glutamine and to a lesser extent, glutamate. The hydrolysis of both the compounds and the control were therefore examined for the glutaminase activity.

Cells were grown to mid-log phase and the glutamine concentration in the culture nitrogen source was determined. The glutamine concentration was then determined using the phenol-hypochlorite procedure of Gustin and Varna. L-glutamine was used as the standard substrate.

The assay containing the cells was incubated with glutamine and glutamate for 2 hours. Cells grown on glutamine showed 80% of the activity of cells grown on glutamate. Cells grown on glutamate showed very little activity indicating the enzyme was specifically located.

Since the activity of the glutaminase activity was present in cells grown on glutamine (which is also used as a deaminating substrate) and glutamate, the presence of glutamine in the cell media is postulated as growth media.
(EC 3.5.1.2)' was postulated to deplete glutamine (Hartman 1973). The enzyme switched off the major biosynthetic pathways thereby conserving energy. Glutaminase was also reported in the bacteroids of *L. angustifolius* and *Glycine max* nodules (Robertson *et al.* 1975, Streeter 1977). Extracts of free-living *R. lupini* were found to hydrolyse both glutamine and to a lesser extent, γ-glutamyl hydroxamate. The hydrolysis of both the compounds can be inhibited by L-5-diazo-4-oxonorvaline (Robertson *et al.* 1975). The cultured cells of strain ANU289 were therefore examined for the presence of glutaminase.

Cells were grown to mid-log phase using glutamate or glutamine as the sole nitrogen source and both supernatant and crude extract were assayed for glutaminase activity (Fig. 6.5). The activity was determined by measuring ammonium concentration spectrophotometrically using the phenol-hypochlorite procedure of Gresshoff (1981). L-glutamine was used as a substrate.

The assays containing no glutamine did not show any glutaminase activity. Cells grown on glutamate showed 70% of the activity of cells pregrown on glutamine. The supernatant showed very little activity indicating the enzyme is intracellularly located.

Since the majority of the glutaminase activity was present in cells grown on glutamate (which is also used as a derepressing nitrogen source) the enzyme had a semi-constitutive nature. The lack of activity in the absence of added glutamine suggested a low level of endogenous glutamine in the cells, irrespective of growth media.
Figure 6.5. Kinetics of glutaminase activity in cultured cells of strain ANU289. The cells were pre-grown using glutamate or glutamine as sole nitrogen source. The activity was measured as μmoles of ammonium released mg⁻¹ protein min⁻¹. Triangles are data derived from cells, while squares represent supernatant.
6.5 Isolation of mutants defective in ammonium assimilation: inhibitor studies and nitrogenase activity in some putative mutants

Studies in the previous section clearly demonstrated that both ammonium and glutamine were capable of inhibiting nitrogenase expression. For a definite identification of the pathways involved in the inhibition, it was necessary to isolate mutants with defects in ammonia-assimilation. This section describes such attempts and the initial studies with the putative mutants.

The Tn5 mutagenised cells were enriched (see Section 2.3.2) so as to obtain mutants with defects in either GS or GOGAT. However, of 300 colonies scored (see Table 6.3), none appeared to be defective in GS. However, 6 colonies were unable to use glutamine alone as a nitrogen source. Since the wild-type ANU289 was able to use glutamine as a sole nitrogen source, these mutants may be defective either in GOGAT, glutamine transport or in glutaminase.

L-Azaserine is a structural analogue of glutamine and competes with its uptake as well as apparently blocking the essential sulfahydroxyl groups in the transferases catalysing the transfer of nitrogen from glutamine onto other substrates. It inhibited growth of strain ANU289 on both ammonium and glutamine at a concentration of 1mM. When the mutants were grown in the presence of glutamine with the similar level of azaserine, some of the mutants, for example 289-A-159, 289-A-163 and 289-A-158, had intermediate levels of growth, whereas the mutants such as 289-A-160, 289-A-165, 289-A-167 and 289-A-169 showed good growth.

The nitrogenase activity in vitro of these mutants was then compared with the parent strain. The activity seen in mutants was 2-3 times higher than with parent strain ANU289. These differences may be due
Table 6.3. Growth characteristics of putative mutants in glutamate, ammonium or glutamine as sole nitrogen source

<table>
<thead>
<tr>
<th>strain/isolates</th>
<th>glutamate</th>
<th>ammonium</th>
<th>glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU289</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>289-A-159</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>289-A-160</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>289-A-163</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>289-A-165</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>289-A-167</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>289-A-169</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Gluconate 5g/l was used as carbon source. Gluconate by itself (in absence of a nitrogen source) did not support growth.
Table 6.4. Nitrogenase activity of assimilation defective mutants in in vitro and in planta

<table>
<thead>
<tr>
<th>strains</th>
<th>growth</th>
<th>acetylene reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in gln</td>
<td>gln + Az</td>
</tr>
<tr>
<td>ANU289</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>289-A-154</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>289-A-165</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>289-A-167</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

Acetylene reduction was measured as nmoles ethylene/mg protein after 8 days incubation in mannitol (50mM), succinate (20mM) and glutamate (3mM). Reduced activity in wild type here may be due to presence of antibiotics 50 µg/ml streptomycin. The mutants contained addition 500 µg/ml kanamycin. In planta, the activity was measured as µmoles/hr/gr FW.
to the nitrogen source used in the derepression medium. The siratro nodules produced by wild-type and mutant Rhizobium strains were equally effective in nitrogen fixation.

6.6 Summary

Regulation of nitrogenase activity by nitrogenous compounds in strain ANU289 appeared to be similar to other Rhizobium strains. Studies in this chapter included the growth of this strain on various nitrogenous compounds, the effect of glutamine and ammonium on derepression of nitrogenase activity, measurement of glutamine uptake and glutaminase activity and finally the isolation of some mutants presumably defective in glutamine assimilation.

Strain ANU289 was capable of growth on all nitrogenous compounds tested except methylvamine. The rate of growth varied with each nitrogen source and was influenced by the accompanying carbon source. Some of the nitrogen sources such as yeast extract, acetamide and glutamate supported vigorous growth whereas other compounds namely ammonium, nitrate, and histidine supported moderate growth. No relationship was apparent between the ability of a nitrogenous compound to support growth and the ability of that compound to allow derepression of nitrogenase activity.

The inhibitory effect of glutamine was seen in stationary liquid culture irrespective of whether it was supplied initially in the derepression medium or added after derepression had occurred. Under the latter experimental procedure, however, ammonium showed more inhibition than glutamine. The effect of ammonium on already derepressed nitrogenase activity was studied in rapidly agitated liquid cultures. A 50% inhibition in the rate of activity compared to the control was observed.
after 5 hrs of injecting 5mM NH$_4^+$. Thus, compared to previously studied *Rhizobium* strains, strain ANU289 appeared to be more susceptible to inhibition by ammonium.

The fact that ammonium inhibition was relieved by MSX suggested that the inhibitory effect may be due to glutamine. This was supported by the evidence that strain ANU289 cells possessed a glutamine uptake system and an intracellular glutaminase which may be responsible for breakdown of glutamine. The precise mechanism of the glutamine effect remains unexplained at present.

In contrast to the wild-type, the mutants were unable to utilize glutamine as a sole nitrogen source. While growth of the parent strain ANU289 was inhibited by azaserine, the mutants were capable of varying degrees of growth. More significantly, the mutant isolates exhibited higher nitrogenase activity than the parent. It is also likely that some of the Tn5 mutagenised-enriched isolates may be defective in GS. In view of these preliminary results, precise biochemical characterisations of the mutants would be interesting.
CHAPTER-7

OXYGEN EFFECTS ON IN VITRO AND IN VIVO NITROGENASE ACTIVITY
7. OXYGEN EFFECTS ON IN VITRO AND IN VIVO NITROGENASE ACTIVITY

7.1 Background

At a biochemical level, oxygen exerts multiple effects on nitrogenase synthesis and activity (reviewed by Eady 1981). Traces of oxygen not only repress the synthesis of nitrogenase polypeptides, but also irreversibly inactivate components of the enzyme. In organisms which use oxidative phosphorylation to generate ATP and reductants, effects of oxygen are highly complex. At low oxygen supply rates, nitrogenase activity is limited by ATP and at saturating ATP levels generation of reducing equivalents becomes limiting, whilst at high oxygen supply rates the dissolved oxygen increases and results in a "switch off" of nitrogenase activity.

Nitrogen-fixing organisms have evolved (see review by Gallo 1981) several means for protecting nitrogenase from oxygen. These are:

1. avoidance (e.g. Clostridium pasturianum),
2. by a physical barrier to oxygen (e.g. heterocysts in cyanobacteria),
3. metabolic removal (e.g. Azotobacter),
4. conformational protection (e.g. Azotobacter),
5. by maintaining a constant level of nitrogenase by a balance between nitrogenase synthesis and inactivation of the enzyme by oxygen as in Anabaena, and
6. by facilitated oxygen transport by leghemoglobin as in legumes.

In Klebsiella pneumoniae oxygen is thought to interact with the nifLA regulatory protein complex, which in turn controls transcription of all the other nif operons including genes nifHDK, which are responsible for the synthesis of the Fe and Mo-Fe (α and β) protein components of nitrogenase. The nifL product was reported to act as a negative effector in the oxygen regulatory mechanism specific to nitrogen fixation (Hill et al. 1981). However, oxygen did not specifically influence synthesis of
nitrogenase polypeptides in *R. leguminosarum* bacteroids, though a high concentration (10µM) of oxygen inhibited the activity of the enzyme (K.C. Van den Bos pers. comm.). In contrast, in slow-growing *R. lupini* bacteroids oxygen differentially inhibited synthesis of Fe and Mo-Fe protein components (Shaw 1983). Whether oxygen regulates nitrogenase synthesis in *Rhizobium* in a manner similar to that observed in *Klebsiella* remains to be answered.

Of particular relevance to this study is the oxygen sensitivity of *in vitro* nitrogenase derepression in *Rhizobium* strain ANU289 and the protection of nitrogenase from oxygen in both legume and non-legume (*Parasponia*) nodules. Protection of nitrogenase from oxygen in symbiotic nodules probably occurs at two different levels. Firstly, the factor most responsible for the low $pO_2$ values in the bacteroid-containing central tissue in legume nodules appeared to be the anatomical arrangement of the intercellular air spaces. A layer of cells in the inner part of the nodule cortex, just exterior to the bacteroid containing tissue, that lacked intercellular air spaces (Fig. 7.1) was discovered in leguminous nodules. This was responsible for a $pO_2$ gradient of about 20 kPa across this layer (Tjepkema and Yocum 1974). A similar compact layer of cells without air spaces was observed in *Parasponia rigida* nodules, but not in other nonleguminous plants (e.g. *Alnus*, *Comptonia*, *Casuarina*) which associate with the actinomycete *Frankia* (Tjepkema 1981, Tjepkema and Cartica 1982). Secondly, legumes in their *Rhizobium* containing nodule cells possess a unique group of hemoproteins, the leghemoglobins. Leghemoglobins are able to bind oxygen reversibly (Kubo 1939), and bear similarities with myoglobins and monomeric hemoglobins in molecular weight (Broughton and Dilworth 1973), amino acid sequence (Richardon et al. 1974) and alignment (Hunt 1972) and spectral properties (Appleby 1974). These properties suggested a role for leghemoglobin in the
Figure 7.1. Cross section of siratro (A) and Parasponia (B) nodules.

The compact layer of cells in the inner part of the nodule cortex void of air space is shown by arrows. VB, vascular bundle; BZ, bacteroid zone; C, cortex; VS, vascular strand; L, lenticells.
nodule tissue associated with the maintenance of a steady low $pO_2$ at the bacteroid surface to ensure that the oxygen-sensitive nitrogenase is not inactivated and also to ensure a sufficient supply of ATP for bacteroid nitrogenase activity (Tjepkema and Yocum 1973, Appleby et al. 1975).

However, in Parasponia nodules no trace of any soluble hemoglobin-like compound could be detected by spectrophotometric analysis or by molecular weight comparison studies, although a small amount of soluble hemoprotein with peroxidase activity was isolated (Coventry et al. 1976). Additionally, the bacteroids isolated from Parasponia nodules contained relatively higher amounts of cytochrome aa$_3$ (Appleby et al. 1981), one of the principal terminal oxidases found in free-living aerobically grown rhizobia (Appleby 1969). It was postulated that either the presence of high cytochrome aa$_3$ permitted vigorous uncoupled protective respiration as reported for soybean bacteroids (Appleby 1978) or the nitrogenase in Parasponia-Rhizobium strains was more tolerant to oxygen than other rhizobia.

In view of the above findings the oxygen sensitivity of nitrogenase activity in the Parasponia-Rhizobium strain was studied. It was observed previously that the oxygen requirement for derepression of nitrogenase activity in this strain was methodology specific (Chapter 3). In this chapter, the oxygen requirement for in vitro derepression of nitrogenase activity of strain ANU289 was compared with other 'cowpea Rhizobium' strains, namely 32H1 and CB756. Oxygen sensitivity of nitrogenase activity was also compared in detached Parasponia rigida and siratro nodules containing strain ANU289.

7.2 Methods

The methods of derepression of nitrogenase activity, i.e. stationary liquid culture and rapidly agitated liquid culture were described
in Chapter 2.3. Other related techniques (such as Rhizobium preculture, acetylene reduction, plant culture, etc.) are presented in Chapter 2.

7.3 Oxygen effects on in vitro derepression of nitrogenase activity: background

Oxygen appeared to be the most important factor influencing nitrogenase derepression in rhizobia in vitro. Nitrogenase activity on agar cultures was measured under atmospheric oxygen tension (0.2 atm), which was later found to be optimal for nitrogen fixation (Pagan et al. 1975, Kurz and La Rue 1975, McComb et al. 1975, Rao and Rao 1977). It was presumed that Rhizobium cells growing on the immediate agar surface and covered by a "dome" of other cells were likely to have nitrogenase because of the microaerobic conditions close to the agar surface.

The location of the large, pleomorphic and nitrogen-fixing cells (resembling bacteroids in nodules) within the Rhizobium colony on the solid agar changed with the oxygen concentration in the atmosphere above agar (Pankhurst and Craig 1978, Van Brussel et al. 1979, Kaneshiro et al. 1983). In stationary liquid cultures, maximum nitrogenase activities were obtained with 20% oxygen in the gas phase (Evans and Keister 1976) and on increasing the oxygen concentration to 40%, the nitrogenase was inhibited drastically. Attempts (Pagan et al. 1975) to derepress nitrogenase in rapidly agitated liquid culture under air were unsuccessful. However, Keister (1975) and Tjepkema and Evans (1976) detected nitrogenase activity in liquid cultures grown in the presence of low oxygen and concluded that a consistently low oxygen tension was obligatory for derepression. Oxygen concentrations in the range of 0.06 to 0.36% were effective with several Rhizobium strains tested (Tjepkema and Evans 1976, Keister and Ranga Rao 1976, Agarwal and Keister 1982). In continuous cultures, nitrogenase activity was derepressed only when the dissolved oxygen concentration was
allowed to drop to or below 1 mM (Bergersen et al. 1976, Ching et al. 1981). The optimal level of oxygen in the gas phase differed with carbon and nitrogen sources used in the medium (Bergersen et al. 1976, Agarwal and Keister 1982). Different strains showed variable oxygen optima, all being within a small range generally classified as microaerobic.

It was not known then, whether the lack of activity under aerobic conditions in rhizobia was due to oxygen repression of nitrogenase synthesis, oxygen inactivation of nitrogenase or auto-oxidation of reduced electron donors. Measurement of antigenically cross-reacting material (Bergersen et al. 1976) in aerated continuous cultures of strain 32H1 showed oxygen repression of nitrogenase synthesis. Exposure of a culture with established nitrogenase activity to moderate oxygen concentrations (20-30 µM dissolved oxygen tension) also resulted in inactivation of nitrogenase (Bergersen et al. 1976). Additionally, effects of oxygen on nitrogenase synthesis were observed by pulse labelling derepressed cells in free-living R. japonicum (Scott et al. 1979) and R. lupini bacteroids (Shaw 1983). However, bacteroids of R. leguminosarum (R.C. Van den Bos pers. comm.) did not exhibit oxygen repression of nitrogenase synthesis, although oxygen did inhibit nitrogenase activity. From studies in R. leguminosarum bacteroids, Laane et al. (1978) concluded that the "switch off" of nitrogenase activity occurred from auto-oxidation of flavodoxin hydroquinone depriving nitrogenase of reducing equivalents. On removal of oxygen stress full activity was regained in the absence of protein synthesis.

7.3.1 Comparison of oxygen requirements of Rhizobium strains ANU289, 32H1 and CB756 in liquid stationary cultures

Previously (see Section 3.4.2) 5 to 10% (v/v) oxygen in the gas phase was found to be optimal for the derepression of nitrogenase
Figure 7.2. Comparison of oxygen effects on nitrogenase activity among Rhizobium strains, 32H1, CB756 and ANU289. (A) Acetylene reduction was measured in nmoles of ethylene mg$^{-1}$ protein after 7 days. Arabinose (50mM), succinate (20mM) and glutamate (3mM) were used. Initial cell density was 20% of a RGM30M grown late-logarithmic phase (absorbance 0.56) culture. Data are averages of five replicates and the average standard error is shown. (B) Oxygen consumption over the culture period was monitored. The final oxygen levels in the vials after 7 days of incubation are shown.
Activity in strain B289G. In view of the reported optimum for strain B289G (in stationary liquid culture) being 30% (v/v) by in the gas phase (Cowl et al., 1970), the experiment was designed to determine the optimum oxygen level in the gas phase in various liquid media. These were: Walker's, Medium A, Medium B, and Medium C (Table 7.1). A number of strains, WU 201 and B289G, both inhibited in various liquid media, were selected for this experiment. Walker's medium was found to be most inhibitory to about 90% of the strains tested. The percentage of oxygen uptake from dissolved oxygen (v/v) was determined as an index of the method being used. Extensive previous experience of these cultures was reported using the same indicator dye system. (Whiting and Cowl, 1970). It was not known if the dissolved oxygen concentration (W) throughout the stationary liquid media and 1% apparent activity (O) gradient (at least because compared to other cultures) gave true to scale in each case.
activity in strain ANU289. In view of the reported optimum for strain 32H1 (in stationary liquid culture) being 20% (v/v) O₂ in the gas phase (Evans and Keister 1976), oxygen effects were compared in three Rhizobium strains, namely ANU289, 32H1 and CB756. The effect of different initial levels of oxygen (i.e. 0, 1.5, 10, 20 and 40% (v/v) in the gas phase) was examined in the above strains and the activity in nmoles of ethylene per mg protein after 8 days is plotted (Fig. 7.2A). The oxygen concentration in the vials was monitored over this period and is presented (Fig. 7.2B).

Strain ANU289 was maximally derepressed at 5 to 10% oxygen in the gas phase, whereas the other two cowpea strains, 32H1 and CB756, both exhibited higher nitrogenase activity at 10 and 20% oxygen. Higher oxygen (40% v/v) in the gas phase resulted in very low activity. Oxygen concentration in these vials was reduced to about 80% of the initial oxygen level at the end of the experiment (Fig. 7.2B).

Thus, in these studies, with stationary liquid cultures, strain ANU289 exhibited a narrower range of oxygen optimum than did strains 32H1 and CB756. It was difficult to conclude from these experiments whether this was due to an extreme sensitivity of nitrogenase in Parasponia-Rhizobium to oxygen compared to the other cowpea strains or an artifact of the method being used. Formation of oxygen gradients on agar cultures was reported using the redox indicator dye resazurin (Pankhurst and Craig 1978). It is not known if the dissolved oxygen concentration is the same throughout the stationary liquid medium, but it appears likely that a gradient (of less intense compared to agar culture) may form in such a system.
7.3.2 Oxygen sensitivity in rapidly agitated cultures

In contrast to the stationary liquid culture approach (Section 7.3.1), derepression of nitrogenase activity in rapidly agitated liquid culture provided an easy and satisfactory procedure to study oxygen sensitivity. Although in this method the actual dissolved oxygen was not measured, assuming equilibration, 0.1% (v/v) oxygen at 760 mm of mercury can be calculated to be approximately 1 µM dissolved oxygen. The actual value may be slightly less than the calculated concentration due to utilization by the rhizobia. A requirement for low oxygen in the gas phase was indicated for nitrogenase derepression in rapidly agitated cultures of strain ANU289 (see Section 3.5.1). The specific activity calculated from time course of derepression at different oxygen levels (Fig. 3.6) is shown in Fig. 7.3.

The high specific rates of nitrogenase activity with strain ANU289 in rapidly agitated cultures with 50 mM succinate as carbon source were obtained with 0.2 to 0.25% (v/v) O_2 in the gas phase. With oxygen concentrations either higher or lower than this level, the onset of nitrogenase activity was delayed, subsequently resulting in low specific rates. The oxygen sensitivity for optimal derepression (0.2 to 0.25% v/v) indicated by these experiments was in agreement with data by Keister and Ranga Rao (1976), who had previously used succinate as the sole carbon source to derepress nitrogenase activity in strain 32H1. The oxygen requirement with succinate as a carbon source was always higher than with other carbon sources (data not shown). This was also observed by Keister and Ranga Rao (1976), and may be due to higher oxygen consumption with succinate as was seen in Chapter 5.4 under both aerobic and microaerobic conditions. Lower rates of nitrogenase activity with higher than 0.25% v/v oxygen indicated the degree of stringency required with respect to the oxygen requirement. Thus, in this respect strain ANU289 appeared to be similar to other derepressable Rhizobium strains.
Figure 7.3. Specific rates of nitrogenase activity with strain ANU289 calculated from Figure 3.6. The rate of activity was measured as increase in ethylene after a 12 h interval and was expressed as nmole ethylene mg\(^{-1}\) protein hr\(^{-1}\).
Figure 7.4  Comparison of derepression kinetics between strains 32H1 and ANU289. The medium contained 50mM sodium succinate (C-source) and 3mM sodium glutamate (N-Source), was buffered with HEPES (pH6.8). Experiment was done at 0.2 to 0.25% (v/v) O₂ in the gas phase and with initial cell concentration of 1-2 x 10⁹ cells/ml. The activity was measured as nmoles of ethylene per 400 ml culture flask containing 10 ml of cells.
Fig. 7.4 shows comparative derepression kinetics of nitrogenase activity of strains ANU289 and 32H1 using similar oxygen levels in the gas phase. The activity appeared in both strains after 24 hrs of incubation. With strain ANU289 the rates were linear between 48 to 72 hrs and then declined. In contrast, activity in strain 32H1 was approximately linear after 72 hrs and remained so until 120 hrs, when the experiment was terminated. Similar to stationary cultures (see Section 7.3.1) strain ANU289 was less efficient in terms of both rate and the total amount of acetylene reduced. Such differences in the level of activity, despite almost similar oxygen requirements among Rhizobium strains has been reported (Agarwal and Keister 1982). The present study indicating a requirement of 0.2 to 0.25% (v/v)O\textsubscript{2} for optimal nitrogenase activity in both strain 32H1 and Parasponia-Rhizobium strain adds to this generality.

7.4 Oxygen sensitivity of nitrogenase activity in Parasponia and in vitro detached nodules containing strain ANU289

The literature contains conflicting reports on estimations of maximum activity in relation to pO\textsubscript{2} in several nodulated species. Reported optima for legumes are between 0.2 and 0.6 atm (Mague and Burris 1972, Hardy et al. 1973) and between 0.1 and 0.3 atm for non-legumes (Silver 1971). The non-legume Casuarina cunninghamiana had a broad maximum and retained 75% of its activity at 0.4 atm pO\textsubscript{2} (Bond 1961), whereas Alnus glutinosa (Bond, 1974) was sensitive to values above 0.2 atm. Acetylene reduction by nodulated Parasponia plants, similar to Alnus glutinosa, was low at 0.1 atm pO\textsubscript{2}, but rose rapidly to a maximum near 0.2 atm pO\textsubscript{2}, followed by a sharp decline with further increase in pO\textsubscript{2} (Trinick 1980). After exposure of Parasponia plants to 0.4 atm pO\textsubscript{2} for
Figure 7.5. Comparison of pO₂ sensitivity of nitrogenase activity in detached nodules of *Parasponia rigida* and siratro containing *Rhizobium* strain ANU289. The values are average of 10 replicates. The standard errors are shown as error bars.
Recently, however, soybean plants were shown to adapt their nitrogen-fixing bacteria to phosphorus deficiencies, which explains the inconsistent results from the sharp optimum near 0.2% air solution for P. Other studies have shown that the surface of the soybean root nodules can also be modified by 0% 02. Perhaps parasponia and other species use different optimum air 02. Furthermore, we note that the same experiments do not indicate that air 02 affects nodule activity of any different species of soybean (Bak et al. 1978). Stringent methods involving the measurement of 02 in the environment can help determine the amount of 02 required for maximum nitrogen fixation. The model was based on light and oxygen availability at all stages of nodulation and development.
20 min the recovery pattern for nitrogenase activity during the course of 2 days (Trinick 1980) was similar to the recovery by soybeans (Criswell et al. 1976).

Recently, however, soybean plants were shown (Criswell et al. 1976, 1977) to adapt their nitrogen-fixing activities to altered rhizosphere pO₂, which explains the inconsistent reports. For example, the sharp optimum near 0.2 atm obtained for Parasponia (Trinick 1980) might have been because the plants were grown in agar slopes and the nodules on the surface of the agar were exposed directly to the atmosphere (0.2atm). Perhaps Parasponia grown in pots or in the field may have a different optimum pO₂. Furthermore, in whole plant assay, the plant by indirect metabolic effects may exert a major influence on measurement of activity. Detached nodules, on the other hand, provide a better experimental system to study the effects of oxygen (Sprent 1969, Houwaard 1978). Factors such as temperature (Pankhurst and Sprent 1976) and pC₂H₂ (Bergersen 1970) which have been reported to cause variations in data, can be easily controlled.

Stringency in oxygen requirement for development of nitrogenase activity in free-living ANU289 cultures on the one hand and report of lack of leghemoglobin (Coventry et al. 1976) on the other made oxygen effects on Parasponia nodules more puzzling. The effect of pO₂ was therefore studied in detached nodules from both Parasponia and siratro plants infected by Rhizobium strain ANU289. The data on pO₂ function, recovery and succinate effects are presented in this section.

The nodules were harvested and immediately put into 27 ml scintillation vials with a drop of sterile distilled water. The gas phase in the vial was changed immediately to argon, 10% acetylene with oxygen provided at the appropriate level. Acetylene reduction was measured over a period of 3 hours and results are shown in Fig. 7.5.
Figure 7.6. The effect of immediate exposure of *Parasponia* nodules to 90% (v/v) oxygen for 3 hours and recovery of nitrogenase activity. The vials containing the nodule were evacuated and filled with 20% O$_2$, 10% C$_2$H$_2$ and 70% argon. The activity was measured over 15 h. The mean values of 5 replicates are plotted. The error bar represents the average standard error.

Figure 7.7. Partially submerged detached nodule assay: The *Siratro* nodules were picked and immediately put into vials containing buffer (with 20mM succinate) and the gas phase containing 30% O$_2$ (v/v) and 10% C$_2$H$_2$ (v/v). Nitrogenase activity was measured over a period of 6 hours. The values are average of 10 replicates and represent the rates. The error bar represents the average standard error.
In *Parasponia* nodules low activity was observed at 0% and 10% (v/v) oxygen. The activity rose rapidly at 20% (v/v) oxygen, was maximal at 30% (v/v) and then declined, such that 50% (v/v) oxygen in the gas phase reduced the activity to almost half of the maximal value; 90% (v/v) oxygen completely inhibited the appearance of acetylene reduction in these vials. In contrast, siratro nodules showed a broad optimum of 30-90% (v/v) oxygen, a characteristic of legume nodules. Previously maximal acetylene reduction was reported as high as 0.6 atm (= 60% v/v) oxygen in field grown soybean plants (Mague and Burris 1972). The maximal activity in siratro was lower than that of *Parasponia* in this study, probably due to the older nodules (10 weeks old), whereas *Parasponia* nodules were younger (8 weeks old). This broad pO$_2$ optimum in siratro is undoubtedly due to the presence of leghemoglobin in siratro nodules and the response observed in siratro nodules to increased external pO$_2$ may be explained by the partially oxygenated leghemoglobin (Bergersen 1962, Appleby 1969).

Detached *Parasponia* nodules incubated in 90% (v/v) oxygen did not reduce acetylene. After 3 hours exposure to such O$_2$ tension, the vials were evacuated and filled with 20% (v/v) oxygen, 10% acetylene in argon. The activity was then measured over a period of 20 hours to examine the recovery of nitrogenase activity (Fig. 7.6). Full recovery of activity was gained by 15 hours. This was in contrast to Trinick (1980), who working with other *Parasponia* species and bacterial isolates found that after exposure of these plants to 0.9 atm pO$_2$, it took 31 hours to recover the full activity. The reasons for these discrepancies if significant, are not known, but may be due to the detached nodule method being used in this study or because of species differences.

The suitability of method used here for studies of pO$_2$ effects was tested further by employing the submerged nodule technique of Sprent (1969). The acetylene reduction of siratro nodules partially submerged in media containing 10 mM succinate, 50 mM MOPS, 2 mM MgCl$_2$ was measured
A similar optimum \( pO_2 \) requirement was obtained. The activity was linear over a period of 360 mins.

Thus, these studies demonstrated that detached Parasponia nodules were sensitive to values above 0.3 atm whereas siratro showed a broader \( pO_2 \) optimum and nitrogenase activity was not inhibited even at 0.9 atm \( pO_2 \). Although slightly broader, probably due to the method being used, the oxygen response curve in \textit{P. rigida} was similar to that of other Parasponia species (Trinick 1980) and nonlegumes (Silver 1971). Similarly the oxygen response curve in siratro nodules was broader than other legumes (Mague and Burris 1972). The maximal nitrogenase activity in these studies was in general 50% of those described for siratro in Chapter 5, where the whole plant was assayed. The loss in activity in detached nodules is well known and is probably due to oxygen entry and lack of other plant derived factors.

With the detached nodules led to the conclusion that \( \text{O}_2 \) concentration played a significant role in the development of nitrogenase activity in \textit{Rhizobium} strain ANU289 both in free-living culture and \textit{in planta}. Results from stationary and rapidly agitated liquid cultures, indicated that a consistently low \( \text{O}_2 \) tension was mandatory for the development of nitrogenase activity. This was achieved by an initial concentration 5-10% (v/v) \( \text{O}_2 \) in the experimental flasks in stationary cultures (in contrast to 10 to 20% for other rhizobia such as 32H1 and CB756). In rapidly agitated cultures of strain ANU289 with succinate, oxygen levels of about 0.2 to 0.25% gave the highest specific rates as was the case with strain 32H1 (not shown).
In contrast to rapidly agitated cultures, varying oxygen tensions had differential effects on nitrogenase activity in detached nodules of *Parasponia* and *siratro*. Similar to stationary culture, *Parasponia* nodules exhibited a narrower optimum compared to *siratro* nodules. This was most probably due to the presence of high concentrations of leghemoglobin in *siratro*, but not in *Parasponia* (see Chapter 8 for a detailed discussion). The recovery of activity in nodules exposed to 90% (v/v) oxygen for 3 hours was more rapid compared to that seen by Trinick (1980), this was perhaps because detached nodules were used in this study. The results of detached *siratro* nodules showed that this can be used as a simple experimental system to compare the effect of several compounds on acetylene reduction.

In conclusion, this chapter indicated that nitrogenase activity in strain ANU289 was equally sensitive to oxygen as was strain 32H1. Studies with the detached nodules incited the possibility that the oxygen protection of nitrogenase in *Parasponia* nodules may be via an oxygen carrier protein of low buffering capacity or by cytochrome(s)-mediated exclusion (detailed in Chapter 8).
CHAPTER-8

CYTOCHROMES AND HEMOGLOBINS

IN

SIRATRO AND PARASPONIA NODULES

INDUCED BY STRAIN ANU289
8. CYTOCHROMES AND HEMOGLOBINS IN SIRATRO AND PARASPONIA NODULES
INDUCED BY STRAIN ANU289

8.1. Preface

Further investigations of the oxygen effects on in vitro and in planta nitrogenase activity focused on the mechanisms of protection of nitrogenase from oxygen damage. Hemoproteins such as hemoglobins and cytochromes play a critical role in this protection mechanism and in the supply of oxygen for bacteroid respiratory activity. Thus, this chapter deals with studies on 'cytochromes and oxidases' in cultured cells (Section 8.4), bacteroids (Section 8.5) and in mutants of strain ANU289 defective in oxidase activities (Section 8.6) as well as an analysis of siratro leghemoglobins and identification of a hemoglobin-like compound in Parasponia nodules (Section 8.7).

8.2 General background

Although nitrogenase per se is extremely oxygen labile, some oxygen is essential to meet the demands of energy (ATP) and reducing equivalents (electrons) necessary for nitrogen fixation. As well as being involved in nitrogen fixation, ATP is required by the bacterial cell for a wide range of other metabolic functions. ATP is synthesized either by substrate level phosphorylation or generated as a consequence of passage of electrons from an electron-donor to an acceptor. This process is called oxidative phosphorylation in which the primary electron donor is the oxidisable substrate (for example a carbon source) and the terminal electron acceptors are either oxygen (in aerobic bacteria) or NO$_3^-$, SO$_4^{2-}$ and CO$_3^{2-}$ (as in anaerobic bacteria). To complement the electron transport, the bacterial cell must be able to establish and
maintain a 'proton motive force' across the cell membrane which 'energizes' the membrane for uptake of various compounds.

Three main factors have been implicated in the rapid modulation of nitrogenase activity in vivo. These are Mg ATP/ADP ratio; supply of reducing equivalents (influenced by membrane potential) and maintenance of sufficiently low dissolved oxygen concentration (see Eady 1981). There is, however, controversy in regard to which of these is more important in nitrogen fixation. Some reports indicated that nitrogen fixation in legume nodules was energy restricted due to the limited availability of photosynthate from the plant (Streeter 1974, Hardy and Havelka 1976, Bethlenfalvay and Phillips 1977). A correlation between nitrogenase activity and ATP/ADP ratio in vivo has been shown in A. vinelandii (Haaker et al. 1974), R. japonicum bacteroids (Appleby 1974) and R. leguminosarum bacteroids (Laane et al. 1978). In these instances the electron transport pathways which were not coupled to ATP formation did not support nitrogen fixation (Appleby et al. 1975, Bergersen and Turner 1975 a, b). However, a contrasting interpretation was reported by Laane et al. (1978, 1979) who proposed that their data were more compatible with nitrogenase activity being controlled by the supply of electrons rather than by the ATP/ADP ratio. It was shown in R. leguminosarum bacteroids that the 'proton motive force' across the cell membrane was involved in the generation of reducing equivalents for nitrogenase (Laane et al 1979). The rate of oxygen supply affected the degree of membrane energisation, particularly the electrical component (ϕ), which in turn affected nitrogenase activity.

Thus appropriate oxygen supply was crucial in producing the 'micromilieu' for nitrogenase functioning without the concomitant inactivation of the enzyme. Several studies have expounded the means by which this fine balance is achieved particularly in nodule bacteroids. Appleby (1969 a, b) demonstrated that there existed multiple potential
oxidation pathways (Fig 8.1) in both cultured and bacteroid forms of \textit{R. japonicum}. A similarly branched electron transport chain recently was reported in \textit{R. trifolii} (de Hollander and Stouthamer 1980). The cytochromes $aa_3$ and $o$ which normally function as terminal oxidases in air grown rhizobia, were replaced with cytochromes P-450, c552 and c554 (Appleby 1969 a,b). Furthermore, each nitrogen fixing bacteroid was surrounded by a concentrated solution of leghemoglobin, with an intracellular oxygen pressure of about $0.006\text{mm Hg}$ or $10\text{nM}$ oxygen.

It facilitated oxygen diffusion to the bacteroid surface (Wittenberg 1970). A positive correlation between concentration of leghemoglobin and nitrogenase activity and the association of the former with effective and ineffective oxygen uptake led Wittenberg et al. (1974) to postulate two distinct terminal oxidases in bacteroids. An oxidase which accepted leghemoglobin-bound oxygen (at a low stabilized tension) was supposed to be more efficient in electron transport (ATP generation), thus supporting better nitrogenase activity. The other oxidase of higher oxygen affinity accepted free dissolved oxygen in the absence of leghemoglobin, and was less efficient in ATP production and thus unable to support nitrogen fixation.

The experimental support for these two oxidases was provided from their differential inhibition by cyanide ($\text{CN}^-$) (Appleby et al. 1975) and 'no gas phase' experiments of Bergersen and Turner (1975 a, b). The leghemoglobin-dependent oxidase system operated only at very low $O_2$ pressure with an optimum near $40\text{nM}$ oxygen coincident with half-saturation of leghemoglobin with oxygen. Oxygen consumption at more than $1\mu\text{M}$ ambient oxygen concentration was independent of leghemoglobin and supported nitrogenase activity only weakly. Later cytochrome P-450 was implicated in the effective phase of oxygen uptake in both \textit{R. japonicum} (Appleby et al. 1975) and \textit{R. lupini} (Kretovich et al. 1973) and was
Figure 8.1. A schematic branching of possible oxidative pathways in rhizobia. The scheme is based on the following studies:

1. Appleby (1969a),
2. Appleby (1969b),
3. Kretovich et al. (1973),
4. de Hollander and Stouthamer (1980) and

(?) Need further substantiation.

* Only observed in cultured cells of one strain of R. japonicum.

** Cyt-P450 is found in bacteroids of some Rhizobium strains and probably in trace quantity in cultured cells of these strains.
suggested to act as a terminal oxidase in the presence of leghemoglobin. The fact that cytochrome P-450 was essential for nitrogen fixation in these bacteroids, was supported by several lines of evidence: (1) correspondence between nitrogen fixation and synthesis of P-450 (Kretovich et al. 1972, Matus et al. 1973), (2) its absence in ineffective lupin nodules (Matus et al. 1973), (3) its absence in cortical R. japonicum cells (Appleby 1969b, as well as (4) inhibitor studies of Kretovich et al. (1974), Melik-Sarkisyan et al. (1974) and Appleby et al. (1975). Additionally in R. lupini, cytochrome P-450 was shown to participate in the peroxide oxidation (Melik-Sarkisyan et al. 1979) of unsaturated fatty acids in the bacteroid membrane. It was suggested that this in turn regulated the state of the membranes which influenced nitrogen fixation.

Relatively little is known regarding the electron transport components and oxidases in bacteroids in absence of leghemoglobin. Under oxygen limited conditions cytochrome a of low redox potential was proposed to act as a major oxidase (Meyer and Jones 1973 a, b; Jones 1973). In contrast under excess oxygen conditions, but in the absence of leghemoglobin the bacteroids had a second defence against oxygen called 'protective respiration'. These bacteroids possessed a CO-insensitive cytochrome c oxidase (Bergersen and Turner, 1973); respiration was un-coupled in this case.

At the onset of this study, a paradoxical situation regarding oxygen supply in the Parasponia nodule existed (see Section 7.1) because of the report of the lack of a soluble legume-type leghemoglobin in nitrogen-fixing Parasponia nodules (Coventry et al. 1976). Yet the bacterium was capable of eliciting leghemoglobin synthesis in nodules on roots of several legumes (Trinick 1980). Additionally, the discoveries such as a compact cortical layer (Tjepkema 1981) and the high cytochrome
aa3 content (Appleby et al. 1981) and subsequently the presence of an oxygen carrier-hemoprotein (Appleby and Trinick 1982) in the cytosol fraction have permitted several speculations regarding the possible oxygen protection mechanisms. One possibility was that the bacteria in the Parasponia nodule were relatively more tolerant to high oxygen concentrations as compared to other rhizobia. The possible presence of cytochromes aa3 and o in Parasponia bacteroids implied either these acted as terminal oxidases permitting nitrogen fixation at moderately high oxygen concentrations or they were involved in 'uncoupled protective respiration' (Appleby 1978). Studies in Chapter 3 (see Sections 3.4.2 and 3.5.1) and Chapter 7 (Sections 7.3.1 and 7.3.2) showed that the Parasponia-Rhizobium strain ANU289 was equally sensitive to oxygen as other 'cowpea rhizobia' for derepression of nitrogenase activity. These, however, were in vitro studies under microaerobic conditions and although nitrogenase was active, it was not necessarily representative of the 'natural' symbiotic state of the bacterium as found in the nodule. For this reason it was thought appropriate to confirm the 'oxygen sensitivity' observation in the symbiotic state. In part this was done with detached nodules of Parasponia and siratro (see Section 7.4) in which an optimum for pO2 requirement ranging from 0.2 to 0.4 atm pO2 was seen in Parasponia nodules. This suggested a definite mechanism for oxygen transfer to the bacteroids in these nodules.

This chapter focuses on the above aspect and extends these symbiotic studies by (i) an analysis of the electron transport components of cultured Parasponia-Rhizobium strain ANU289 and its mutants, (ii) a comparison of electron transport components of siratro and Parasponia bacteroids and (iii) initial studies to partially characterise the hemoglobinins in siratro and possibly in Parasponia nodules.
8.3 Methods

The techniques of bacteroid isolation and bacteroid fractionation (2.3.5), particle preparation and analysis of spectral properties (2.3.6) are described. The isolation of mutants defective in the electron transport system is shown in Section 2.3.3. The procedures for isolation and partial purification of siratro leghemoglobin and Parasponia hemoglobin are presented in 2.4.4. A summary of these methods is presented in Fig. 8.2.

8.4 Presence and distribution of cytochromes in Parasponia-Rhizobium strains - Background

The aerobic respiratory chain of several Rhizobium species showed many similarities to the respiratory chain of mammalian mitochondria both in the types of respiratory carriers present and in its sensitivity to inhibitors (John and Whatley 1975). However, many features not normally found in mitochondria were also observed and it was suggested that these were due to the terminal branching of the respiratory chain as found in many other bacteria (Haddock and Jones 1977).

Smith (1949) first described the aerobic respiratory chain of cultured cells of R. japonicum and demonstrated the presence of cytochrome aa₃, b and c. This was later confirmed by qualitatively similar patterns in R. japonicum (Appleby 1969b), R. lupini (Kretovich et al. 1973), R. leguminosarum (Kretovich et al. 1973) and R. trifolii (de Hollander and Stouthamer 1979). The respiration of succinate by air-grown cells was substantially inhibited by CO and a photochemical action spectrum showed the function of both cytochromes aa₃ and o as terminal
Figure 8.2 Summary of methods used for comparison of nodule hemoproteins of siratro and Parasponia infected by Rhizobium strain ANU289

Grinding of nodules in buffer

\[ \rightarrow 200g(10\text{min}) \]

Post-nuclei supernatant on top of 35% w/v sucrose

\[ \rightarrow 17000g(15\text{min}) \]

Bacteroid pellet

\[ \rightarrow \]

Sucrose density gradient centrifugation

\[ (57-52-50-45\% \text{ w/w}) \]

\[ \rightarrow \]

Fractionation with 65% w/w sucrose

\[ \rightarrow \]

Bacteroids from different fractions resuspended in PB 6.8 at 25% wet w/v

DNase (2mg/l)

\[ \rightarrow \]

MgCl₂ (2mM)

\[ \rightarrow \]

French Pressure Cell 2×

\[ \rightarrow 17000g(30\text{min}) \]

Supernatant

\[ \rightarrow 187000g(2h) \]

Particles

Supernatant

\[ \rightarrow \]

Spectral analysis

Supernatant

Ammonium sulphate fractionation

\[ \rightarrow \]

concentrated (Diaflo PM 10)

\[ \rightarrow \]

DEAE cellulose

\[ \rightarrow \]

Fractions from a linear gradient of 15-150mM acetate buffer

Analytical

\[ \rightarrow \]

SDS PAGE electrophoresis
oxidases (Appleby 1969 b). In addition to $aa_3$ and $o$, a third possible oxidase, cytochrome $d$, was mentioned (Kretovich et al. 1973).

Ubiquinone formed a link between the NADH dehydrogenase and the cytochrome part of the electron transport chain in *R. japonicum* (Daniel 1979). Cytochrome spectra and proton translocation stoichiometries provided evidence for the characterisation of the electron transport chain in *R. trifolii*. Similar to *R. japonicum* cultured cells, the branching point was shown to be cytochrome $b$-555 (de Hollander and Stouthamer 1979, 1980).

Environmental factors such as aeration and nitrate were implicated for the variation in biosynthesis of *Rhizobium* hemoproteins. Kretovich et al. (1973) compared the cytochrome pattern of the effective and ineffective strains of *R. leguminosarum* in aerobic and microaerobic conditions. The pattern remained the same under aerobic conditions, whereas a difference mainly in cytochrome $a$ was observed in microaerobic conditions. The effective strains synthesized cytochrome $a_3$ in trace amounts. In other studies anaerobiosis rather than nitrate was held responsible for the altered cytochrome pattern such as disappearance of cytochrome $aa_3$ and the appearance of CO-reactive P-450 and c552 in anaerobic nitrate grown cells of *R. japonicum* (Daniel and Appleby 1972).

In contrast in free-living *Rhizobium* strain 32H1, the level of $aa_3$ was essentially similar under non-fixing, aerobic as well as nitrogen-fixing conditions, whereas cytochrome $b$- and $c$-content and cytochrome oxidase activity increased 3 to 5 fold under nitrogen-fixing conditions (Evans and Christ 1980).

The role of hemoprotein oxidases in respiration was studied using an alternative approach. This comprised the isolation of mutants defective in their oxidase activity employing the classical 'oxidase test'. Mutants were used to dissect the respiratory chain in
Rhodopseudomonas capsulata (Marrs and Gest 1973), and Paracoccus denitrificans (Willison and John 1979). El Mokadem and Keister (1982) for the first time isolated cytochrome c and aa₃ deficient mutants in R. japonicum. Some of these mutants were infective but formed ineffective nodules (nod⁺ fix⁻). This approach was used in the present study to determine the role of oxidases.

8.4.1 Electron transport components of cultured cells of strain ANU289, 32H1 and CB756

Rhizobium strains ANU289, 32H1 and CB756 (see Section 2.1) all capable of nodulating Parasponia were examined for their cytochrome contents after aerobic culture in TY medium (Table 8.1). Strains 32H1 and CB756 exhibited almost identical spectra (Fig. 8.3a).

In reduced minus oxidised difference spectra, the insoluble particulate fraction of all these strains exhibited similar spectra, typical of many bacteria which contain cytochromes aa₃ (λₘₐₓ 603), b (λₘₐₓ 560) and c (λₘₐₓ 554-551). These room temperature spectra were similar to that observed by Tazimura and Watanabe (1964) and Appleby (1969b). In cultured cells of strain 32H1, Evans and Christ (1980) reported cytochrome c was present in larger amounts than cytochrome b. But in the present study, consistently all strains showed more of cytochrome b compared to cytochrome c. However, when strain ANU289 was grown on gluconate-yeast extract medium, the cytochrome c was found in higher amounts than cytochrome b (Fig. 8.3b). The same was the case with bacteroids (see Section 8.5). Thus, media in which the cells were grown may influence the cytochrome composition. Similar to all previous observations, cytochrome b and aa₃ remained particle bound following cell disruption.
### Table 8.1. Relative cytochrome concentration of *Rhizobium* strains ANU289, 32H1 and CB756 in aerobically grown cells

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hemeprotein content (nmoles of Heme/g protein)</th>
<th>Reduced minus oxidised</th>
<th>CO reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>aa</em>&lt;sub&gt;3&lt;/sub&gt;</td>
<td><em>b/o</em></td>
</tr>
<tr>
<td>ANU289</td>
<td></td>
<td>230</td>
<td>690</td>
</tr>
<tr>
<td>32H1</td>
<td></td>
<td>220</td>
<td>670</td>
</tr>
<tr>
<td>CB756</td>
<td></td>
<td>390</td>
<td>740</td>
</tr>
</tbody>
</table>

The cells were grown on TY medium (Section 2.2.2) and membrane particles were prepared as in Section 2.3.6.

++ present but not quantifiable.

+ present in trace quantity.

− not identifiable.
Figure 8.3a. Cytochrome spectra of cultured cells of strain ANU289 grown aerobically in gluconate yeast extract medium.

(A) reduced minus oxidised difference spectrum of particulate fraction, (B) reduced plus CO minus reduced difference spectrum of particulate fraction. (C) reduced minus oxidised spectrum of soluble (supernatant) fraction, (D) reduced plus CO minus reduced difference spectrum of soluble (supernatant) fraction.

In reduced minus oxidised spectra, cytochromes $a_3$, $b$ and $c$ are identified by the $a$-maxima at 600-605nm, 560nm and 551nm respectively. In CO-difference spectra, $a_3$ is identified by the absorption maxima at 596nm and 433nm and minima at 606-610nm and 447nm. CO reactive cytochrome $o$ has a spectrum with absorption maxima at 538nm and 547nm and Soret at 416-517 and the minima at 574nm. CO reactive cytochrome $c$ is identified by a maxima at 539 and 569nm, Soret at 415nm and a minima (or shoulder in opposite) at 552nm.
Figure 8.3b. Cytochrome spectra of cultured cells of cowpea-Rhizobium strains CB756 and 32H1. (A) reduced minus oxidised difference spectra and (B) reduced plus CO minus reduced difference spectra. The identification of cytochromes and CO reactive pigments was as described in Fig. 8.3a legend.
A

B

A = 0.04

A = 0.04

nm

nm
CO reactive components

Several CO-reactive components were identified in the particulate fraction of cultured cells of *R. japonicum* strain 505 (Appleby 1969b). These were cytochrome $a_3$, $o$, and $P\!-\!428$, a cytochrome identified only by its Soret band. The supernatant contained a pigment with many properties of hemoglobin and was called 'Rhizobium hemoglobin'. In *R. japonicum* 61A76 El Mokadem and Keister (1982) failed to observe $P\!-\!428$ but found a CO-reactive $c$-type cytochrome in both particulate and soluble fractions.

In strains ANU289, 32H1, and CB756, the particulate CO-reactive components were mainly cytochrome $a_3$ and cytochrome $o$ (characterised by a minima at 562-465nm and maxima at 574-438nm and a Soret peak at 417-418nm). A particulate $P\!-\!420$ was observed in 32H1 and CB756 but not in ANU289. However, the latter strain, when cultured in gluconate-yeast extract medium appeared to contain a soluble $P\!-\!420$ with Soret maxima at 422nm (Fig. 8.3b). Similarly, neither the particulate nor the soluble cytochrome $c$ was CO-reactive in strains 32H1 and CB756 but they were CO-reactive in strain ANU289. The so-called *Rhizobium* hemoglobin was not observed in cultured cells of any strain. Whether cytochrome $aa_3$ and $o$ acted as terminal oxidases was not determined, but from previous studies and their auto-oxidisability it was presumed that in aerobically grown cells these along with cytochrome $P\!-\!420$, were the terminal oxidases.

Low temperature difference spectra

At low temperatures (-196°C), the difference spectrum of particles of ANU289 showed cytochrome $b$ resolved into two components: one having an $a$ peak at 555.0nm and the other an $a$ peak at 559.5nm (data not shown). This was previously observed by Appleby (1969a) and Kretovich et al. (1973). Both of these reports associated the 559-component with cytochrome $o$ and the 556 component with cytochrome $b$. However, based on mutant studies, El Mokadem and Keister (1982) suggested that the
555-component was cytochrome $a$. The single $a$ peak for cytochrome $c$ (548 nm at -196°C and 551 nm at 20°C) suggested the presence of only one cytochrome $c$ component in strain ANU289. This was further supported by the $a$ peak of soluble cytochrome $c$ at 551 nm (at 20°C).

8.5 Presence and distribution of cytochromes in *sinatro* and *Parasponia* bacteroids

Next to leghemoglobin, the dominant hemoproteins of nitrogen-fixing nodules, are the bacteroid cytochromes. The study of bacteroid cytochromes is important because of the likelihood that at least some more oxygen carriers, terminal oxidases or intermediate electron carriers in the bacteroid's oxidative phosphorylation. Furthermore, the presence of leghemoglobin, because of its oxygen transport activity, differentially modulates the transformation of the cytochrome pattern when vegetative bacteria differentiate to become bacteroids. For example, in *R. japonicum* (Appleby 1969a, Daniel and Appleby 1972) and *R. lupini* (Kretovich et al. 1972, 1974, Matus et al. 1973, Melik-Sarkisyan et al. 1974) bacteroids, the respiratory chain was characterised by the presence of several oxidases namely cytochrome $c_{552}$, one or more cytochrome $b$, cytochrome $P$-$428$, and $P$-$450$ (see Section 8.2 for a detailed review, also Fig. 8.1). In contrast, in *R. leguminosarum*, the bacteroids showed an oxidase pattern with a trace of $aa_3$ and a much dominating cytochrome $o$ and $P$-$420$ rather than $c_{552}$ and $P$-$450$; and remained efficient at oxidative phosphorylation at more than 1µM dissolved oxygen.

The reported disappearance of cytochrome $a$ from the bacteroids of *R. japonicum* 505 (Appleby 1969a) is another example. This was also found in lupin nodules by Kretovich et al. (1972). Additionally, this author also reported that the rhizobia from ineffective nodules (containing little or
Table 8.2. Comparison of cytochrome concentration in siratro and Parasponia bacteroid preparations

<table>
<thead>
<tr>
<th>cells derived from</th>
<th>Particulate</th>
<th>Soluble</th>
<th>c552</th>
<th>P-420</th>
<th>CO reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siratro nodule</td>
<td>aa3</td>
<td>b</td>
<td>c</td>
<td>c</td>
<td>Particulate soluble</td>
</tr>
<tr>
<td></td>
<td>106</td>
<td>730</td>
<td>535</td>
<td>280</td>
<td>25</td>
</tr>
<tr>
<td>Parasponia nodule</td>
<td>trace</td>
<td>1060</td>
<td>520</td>
<td>306</td>
<td>200</td>
</tr>
<tr>
<td>aerobic cultures</td>
<td>230</td>
<td>690</td>
<td>460</td>
<td>110</td>
<td>+</td>
</tr>
</tbody>
</table>

The bacteroids were isolated (Section 2.3.5), membrane particles were prepared (Section 2.3.6) and the concentration of hemeproteins were derived from the cytochrome spectra analysis.

+ trace amount.
++ present in relatively more amounts, not quantifiable.
- not detectable.
no leghemoglobin) contained a small amount of cytochrome a. The increase in cytochrome a content of bacteroids from these nodules compared to those of effective nodules was interpreted to be either due to the prevalence of a more aerobic environment in ineffective nodules or a specific suppression of cytochrome a synthesis in the presence of leghemoglobin. Consistent with this hypothesis by growing the cells in microaerobic conditions (Kretovich et al. 1973, Avissar and Nadler 1978), an increase in cytochrome a content was obtained. However, recently cytochrome aa₃ was reported in bacteroids of R. leguminosarum, Parasponia-Rhizobium NGR231 (Appleby et al. 1981) and R. japonicum 61A76 and several other strains (Keister et al. 1983). Furthermore, the level of cytochrome aa₃ was constant under aerobic and microaerobic (nitrogen-fixing) in vitro conditions (Evans and Christ 1980). Thus, in summary, the cytochrome aa₃ situation is ill-defined and highly controversial in view of conflicting results and interpretations.

Because of the atypical cytochrome spectra of aerobically grown cells of strain ANU289, 32H1 and CB756 (Section 8.4) and differences in oxygen sensitivity seen in siratro and Parasponia nodules infected by strain ANU289 (see Section 7.5), this section compares the cytochrome content of bacteroids isolated from both siratro and Parasponia nodules. Since the bacteroids in legume and non-legume nodules did not represent a homogeneous cell population, these were fractionated by gradients and the distribution of cytochromes in these fractions was ascertained.

8.5.1 Cytochrome contents in total bacteroid preparations

The preparation of bacteroids and analysis of their cytochrome composition was done in this study as described by Keister et al. (1983). The cytochrome content of the particles and supernatant was calculated from dithionite reduced minus air or Fe(CN₆)³⁻ oxidised difference spectra. Table 8.2 shows the comparison of cytochromes in total
Figure 8.4. Comparative cytochrome spectra of total bacteroid preparations of strain ANU289, isolated from siratro and Parasponia nodules. (A) reduced minus oxidised difference spectra (B) reduced plus CO minus reduced difference spectra.
bacteroids from siratro and Parasponia nodules infected with Rhizobium strain ANU289. Fig 8.4 depicts the comparative spectra of particulate fraction of siratro and Parasponia bacteroids.

In reduced minus oxidised difference spectra, the concentration of cytochrome aa3 (Amax 603nm) in siratro bacteroids (Fig. 8.4) was about 50% of that of cultured cells (Table 8.2), whereas it was present only in trace amounts in Parasponia bacteroids. Cytochrome b (λmax 561nm) was present in larger amounts in Parasponia compared to those of siratro bacteroids; cytochrome c (max 552nm) content was similar in both bacteroid types. There was a slight increase in b and about a 40% increase in c content in bacteroids compared to cultured cells. A similar increase in cytochrome b- and c-content was previously seen in R. japonicum (Appleby 1969a, b; Keister et al. 1983) and R. lupini (Kretovich et al. 1973, Matus et al. 1973). There was no shift in absorption maxima of the cytochromes in bacteroids compared to vegetative cells.

The CO difference spectra of the bacteroids from both siratro and Parasponia consisted of CO-reactive c552, o and P-420. In siratro the majority of CO-reactive c552 was in a soluble form, whereas in Parasponia, the majority of c552 was in a particulate fraction. In contrast to Parasponia bacteroids, which showed more of possible cytochrome o, siratro bacteroids contained only a trace amount, although cultured cells had shown large amounts. In general it can be concluded that bacteroids contained a much lower amount of observable cytochrome o.

There was essentially no indication of P-420 in the particulate fractions, but there was a soluble P-420 especially in siratro bacteroids. The same was observed in the supernatant of R. japonicum USDA138 (Keister, pers. comm.). The proper identification of this component, however, requires fractionation of the supernatant to make it free from cytochrome c. No peak was observed at 450nm in CO difference spectra of both siratro
and Parasponia bacteroid preparations indicating the absence of cytochrome P-450.

Thus, the bacteroids from siratro and Parasponia nodules showed an oxidase pattern characteristic of R. leguminosarum with much dominating cytochrome P-420, o and c552 and a reduced amount of cytochrome aa3. It is possible that these oxidases in Parasponia bacteroids function well at the presumed moderate oxygen tension in the absence of a classical leghemoglobin. In terms of free oxygen concentration, these nodules may be similar to those of pea (Kretovich et al. 1973, Appleby et al. 1981).

8.5.2 Fractionation of bacteroids from Parasponia and siratro nodules

The differentiation of free-living Rhizobium cells to bacteroids in nodules is characterised by several changes: an increased cell size (Bergersen 1974), cell wall composition (Van Brussel 1977), uptake of metabolites (Werner and Berghauser 1976), DNA content (Van den Bos et al. 1978), variations in enzyme activities (Rigaud et al. 1973, Bishop et al. 1976, Van den Bos et al. 1978, Werner and Stripf 1979) and alterations in the cytochrome pattern (Daniel 1979). In a more detailed study, Ching et al. (1977) separated the bacteroids of soybean into vegetative bacteria, transforming bacteroids and matured bacteroids and showed that the latter were responsible for the majority of nitrogenase activity. These bacteroids were devoid of cytochrome aa3 and contained less cytochrome c oxidase compared to transforming and vegetative bacteria.

Cytochromes aa3 and o (a terminal oxidase in cultured cells, Appleby 1969b), were reported to be present in Parasponia-NGR231 bacteroids (Appleby et al. 1981). A possible involvement of these cytochromes in energy production for nitrogen fixation was suggested in the absence of leghemoglobins in Parasponia nodules (Coventry et al. 1976). Even if a hemoglobin was present (but not detectable), the
Parasponia nodules containing strain ANU289 were poor in oxygen-buffering compared to those of siratro (see Section 7.5). Additionally, the bacteroid from these nodules contained both cytochromes aa3 and o. In an attempt to further characterise these cytochromes, this section examines their distribution in both Parasponia and siratro nodules.

To date three different methods have been used for the isolation of bacteroids from legume nodules, such as (1) differential centrifugation (Bergersen 1958) which allows rapid isolation but the preparations are usually contaminated by plant components, (2) a self-generating percoll gradient (Reibach et al. 1981) and (3) sucrose density gradient centrifugation (Sutton and Mahoney 1977, Ching et al. 1977). Since in Parasponia rigida the nitrogen fixing bacteria remain in the infection threads (Price et al. in preparation) and no attempt was made previously to analytically separate these bacteria, the discontinuous gradient method used for soybean (Keister et al. 1983) was adopted to fractionate the nodule bacteria in both siratro and Parasponia.

The distribution of nodule bacteria in both siratro and Parasponia nodules induced by Rhizobium strain ANU289 is shown in Fig. 8.5. The bacteroid fraction from these nodules separated into four fractions as measured by optical density (680nm). The lightest peak consisted of the plant fraction which was always present in larger amounts in siratro compared to Parasponia. The bacteria were distributed on the interfaces between 45-50% (density = 1.230), 50-52% (density = 1.240) and 52-57% (density = 1.262) in the discontinuous gradient. The quantitative distribution of bacteria in the gradients of Parasponia and siratro was similar. More significantly, the heaviest fraction constituted almost one third of the total nodule bacteria. Cultured ANU289 cells banded entirely at this lower interface. 10g each of nodules yielded approximately twice as much bacteria in siratro compared to Parasponia. Electron microscopy of the different fractions in gradients of siratro and Parasponia.
Figure 8.5. Elution profile of bacteroids (OD 650nm) from siratro (•—•) and Parasponia (▲—▲) nodules after a discontinuous sucrose density gradient centrifugation. Essentially the method of Keister et al. (1983) was followed (see Section 2.3.5). Three major fractions corresponding to tube numbers 11 to 16 (light), 21 to 25 (intermediate) and 27 to 32 (heavy) were obtained. The specific gravity ($\delta_{20}^{\circ}$) of individual fractions was measured with a refractometer and is plotted (■—■)
demonstrated that these fractions were substantially free of mitochondria (Fig. 8.6). The measurement of the diameter of bacteroids from scanning electron micrographs showed that the Parasponia bacteroids of the light (corresponding to matured bacteroids) and intermediate (corresponding to transforming bacteria) fractions were respectively 34% and 21% larger than corresponding siratro fractions, whereas the bacteria in heavy fraction remained the same in both host types.

The lower yield of bacteroids from Parasponia nodules can also be explained from the ultrastructural work with these nodules (Price et al. in preparation). It was estimated that there were fewer bacteria in Parasponia nodules (about 295 per 1000 cubic microns). This is probably due to the Parasponia nodule ultrastructure, in which the bacteria remain confined to the ramifying infection threads. The thread wall may impose a restriction on bacterial multiplication. The similar pattern of distribution of the different bacteroid forms in two different host types reflected that the control of this differentiation is not under plant control but solely due to bacterial genes. These distribution patterns were, however, different from those of soybean (Keister et al. 1983) in as far that a larger proportion (about one-third) of bacteria remained vegetative.

8.5.3 Cytochromes in separated bacterial fractions

The separated bacterial fractions from the discontinuous sucrose gradient (see previous section) were examined for the presence and distribution of cytochromes and the data are presented in Table 8.3.

The reduced minus oxidised difference spectra of the various bacterial fractions of siratro and Parasponia nodules showed no significant differences in concentration of major CO insensitive cytochromes b and c when compared with the total bacterial preparations (see Table 8.2). The soluble fraction of cytochrome c was present in
Table 8.3. Cytochrome content in different fractions of nodule bacteria of siratro and Parasponia

<table>
<thead>
<tr>
<th>fractions</th>
<th>hemeprotein content (nmoles of Heme/g protein)</th>
<th>CO reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>particulate</td>
<td>soluble</td>
</tr>
<tr>
<td></td>
<td>aa₃ b c c</td>
<td>particulate</td>
</tr>
<tr>
<td>Parasponia:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>- 190 316 230</td>
<td>60 52</td>
</tr>
<tr>
<td>II</td>
<td>- 300 320 250</td>
<td>50 45</td>
</tr>
<tr>
<td>III</td>
<td>+ 400 480 230</td>
<td>90 100</td>
</tr>
<tr>
<td>Siratro:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>+ 590 450 70</td>
<td>90 20</td>
</tr>
<tr>
<td>II</td>
<td>+ 570 570 230</td>
<td>60 20</td>
</tr>
<tr>
<td>III</td>
<td>130 550 590 60</td>
<td>100 20</td>
</tr>
</tbody>
</table>

The bacteroids were isolated from the nodules and fractionated. Each individual fraction was collected and the cell-free extract was prepared for cytochrome spectral analysis. P-420 was only observed in soluble fraction. -, not detectable; +, trace; and +, ++ relative amounts present.
Figure 8.6. Scanning electron micrographs of the different fractions from the discontinuous sucrose gradients (see Section 8.5.2) of siratro and Parasponia bacteroids. P = Parasponia, S = siratro, Fractions 1, 2 and 3 respectively correspond to 'matured bacteroids', 'transforming bacteroids' and 'vegetative bacteria'. Bacteria from these fractions were washed and passed through a 0.6 micron nucleopore filter and the filters were prepared for scanning. The magnification is x6000.
larger amounts in the bacterial fraction of Parasponia than of siratro. In siratro most of the cytochrome $a_3$ was present in the heavy fraction, less in the intermediate fraction and only in trace in the light fraction. In Parasponia no cytochrome $a_3$ was detected in the light fraction but trace quantities were seen in the intermediate and heavy fractions.

The CO difference spectra showed no major differences in the concentrations of CO reactive pigments in various fractions of siratro and Parasponia, except for cytochrome o, which appeared to be present in larger quantities in 'matured bacteroids' of Parasponia compared to that of vegetative and transforming bacteroid fractions.

Thus, in regard to cytochrome $a_3$, the results of the present study are in agreement with those derived from soybean, where most of the cytochrome $a_3$ was present in vegetative bacteria and the matured bacteroids contained the least. Furthermore, it was observed in these fractions that the decrease in cytochrome $a_3$ was associated with concomitant increases in cytochrome o. This suggested that cytochrome o was probably one of the major terminal oxidases in Parasponia bacteroids.

8.6 Studies with mutants of Rhizobium strain ANU289 defective in electron transport system.

Whether or not the cytochromes $a_3$ and o (see sections 8.4 and 8.5) act as terminal oxidases in strain ANU289 and whether there exists a relationship between presence or absence of these cytochromes and nitrogen fixation was investigated using oxidase deficient mutants as was previously done in R. japonicum strain 61A76 (El Mokadem and Keister 1982). Studies on the isolation of oxidase deficient mutants, the spectral characterisation and the ability of these mutants to fix nitrogen in vitro as well as in planta are described in this section.
8.6.1 Isolation of mutants defective in electron transport components

Keilin (1966) described the 'Nadi Reaction' which has been empirically used as an 'oxidase test'

\[ \text{α naphthol} + \text{dimethyl-p-phenylenediamine} + \text{oxygen} \rightarrow \text{colourless} \]
\[ \text{cytochrome c} \rightarrow \text{cytochrome oxidase} \]
\[ \text{indophenol blue} + \text{water} \rightarrow \text{blue colour} \]

The mixed organic reactants (termed 'Nadi' reagent, an acronym composed of the first two letters of each chemical name) are colourless in the reduced state and are rapidly oxidised to coloured forms by 'oxidase positive' species but not by 'oxidase negative' species.

When mutagenised colonies (either NTG induced or Tn5 insertion mutants) were subjected to the oxidase test (see primary screening procedure, Section 2.3.3), twelve different mutant isolates with possible altered staining characteristics were obtained. These were checked for their antibiotic resistance and phenotypic markers and then put into the secondary screening procedure, which involved a 'Nadi Reaction' with aerobically grown cells in liquid culture (see Section 2.3.3). The results of these tests are shown in Table 8.4.

The parent strain ANU289 grew normally and produced maximal oxidase activity as measured by 'Nadi reaction'. In the mutant isolates, the oxidase activities were reduced several fold. No mutant isolate, however, completely lacked oxidase activity. The control buffer without cells showed no activity. The secondary screening procedure thus confirmed that the mutant isolates lacked one or more of the oxidases.
8.6.2 Relative aerobic growth, whole cell and \textit{in planta} nitrogenase activity of the putative Ets mutants

The relative growth of various mutants grown in BMM is shown in Table 8.4. Some of these possible mutants such as Ets1, Ets3, Ets6, Ets7 and Ets8 grew normally in contrast to Ets5, Ets11, Ets12 and Ets13 in which growth was reduced. The growth characteristics of the mutants observed in this study are in agreement with \textit{R. japonicum} mutants (El Mokadem and Keister 1982). Inability to isolate complete oxidase deficient mutants along with the observation of normal growth characteristics of most of these mutants corroborated the possibility of multiple oxidation pathways, typical of \textit{Rhizobium} species.

Table 8.4 also shows the nitrogen fixation characteristics of these mutants both in culture and in siratro. In culture, the acetylene reduction by the parent strain ANU289 (641 n moles ethylene/vial) was comparable to previous studies. Whereas, in the mutant isolates, the total nitrogenase activity was reduced by 2 to 6 fold. Ets1, Ets11 and ETS13 showed only one-sixth of wild type activity. In Ets3, Ets4, Ets5 and Ets12 the activity was reduced by about 50%. Moreover, when the mutant isolates were tested for nodulation on siratro and Parasponia, all were capable of nodulation. The \textit{in planta} nitrogenase activity measured after 6 weeks showed that the majority of the isolates were either partially effective (nod$^+$fix$^+$) such as Ets 2, Ets4, Ets5, Ets11, Ets12 and Ets 13) or fully ineffective (nod$^+$fix$^-$, c.f. Ets1 and Ets7). Some of these putative mutants (Ets3, Ets8, Ets9 and Ets13) were, however, nod$^+$fix$^+$ as was the parent strain ANU289.

Ineffectiveness of \textit{R. japonicum} mutants defective in electron transport components was previously reported by El Mokadem and Keister (1982). The partial effectiveness of the majority of the mutants in the present study may be due to a transposon being used as the mutagen (which causes only a single mutation, whereas multiple mutations may be necessary).
Table 8.4. Secondary screening for Ets defective mutants, their relative aerobic growth and whole cell and in planta nitrogenase activity

<table>
<thead>
<tr>
<th>mutants</th>
<th>mutagen used</th>
<th>OD (750 nm)</th>
<th>Klett reading</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU 289</td>
<td></td>
<td>0.724</td>
<td>325</td>
<td>641 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ets 1</td>
<td>NTG</td>
<td>0.194</td>
<td>320</td>
<td>134 +</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ets 2</td>
<td>NTG</td>
<td>0.201</td>
<td>290</td>
<td>NT</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Ets 3</td>
<td>Tn5</td>
<td>0.198</td>
<td>335</td>
<td>200 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ets 4</td>
<td>Tn5</td>
<td>0.208</td>
<td>365</td>
<td>386 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ets 5</td>
<td>Tn5</td>
<td>0.240</td>
<td>230</td>
<td>258 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ets 6</td>
<td>Tn5</td>
<td>0.332</td>
<td>385</td>
<td>NT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ets 7</td>
<td>NTG</td>
<td>0.486</td>
<td>365</td>
<td>NT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ets 8</td>
<td>NTG</td>
<td>0.345</td>
<td>350</td>
<td>NT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ets 9</td>
<td>Tn5</td>
<td>0.305</td>
<td>375</td>
<td>NT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ets 11</td>
<td>Tn5</td>
<td>0.182</td>
<td>215</td>
<td>102 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ets 12</td>
<td>Tn5</td>
<td>0.174</td>
<td>135</td>
<td>252 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ets 13</td>
<td>NTG</td>
<td>0.302</td>
<td>279</td>
<td>101 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The cells were grown in BMM, a medium which produces minimum polysaccharides

OD. readings are average of two experiments.

A. in vitro fixation. nmoles ethylene/vial.

B. nodulating ability of siratro.

C. in planta fixation (siratro).

NT. not tested.
8.6.3 Relative cytochrome concentration of possible Ets mutants in aerobically grown cells

Eight mutant isolates were characterised for their spectral properties (Table 8.5). The reduced minus oxidised spectra of the mutants showed that Ets1 and Ets7 had only trace amounts of cytochrome aa3 and the amount of aa3 was reduced by about 50% in Ets5, Ets6 and Ets12. Similarly cytochrome c was present only in trace quantity in Ets1. The cytochrome content was reduced in all isolates except Ets8 and Ets13. Two isolates namely Ets5 and Ets12 had a decreased content of cytochrome b. An increased cytochrome b content compared to cytochrome c observed in parent strain ANU289 in aerobically grown cells was also observed in all these mutants. Since low temperature spectroscopy was not performed in the case of the mutants, the cytochrome c phenotype of the mutants remains obscure.

The CO-difference spectra of strain ANU289 had indicated primarily three CO reactive pigments. These were cytochromes c552, o and P-420. Cytochrome c552 was present in trace quantity in Ets1, Ets5, Ets8 and Ets12. A particulate P-420 which has also been described as a possible terminal oxidase (Appleby 1969b) was absent in Ets6 and Ets13 (as it is in strain ANU289) but was present in all other isolates in varying amounts.

Cytochrome o (as determined from CO difference spectra) was present in all mutant isolates to varying degrees, although in lower amounts than observed with the parent strain.

In preliminary studies of these eight mutants which were spectrally analysed, the gross cytochrome composition of each was different from others. Three of these Ets1, Ets7, Ets8 were NTG induced mutants. Ets1 characterised by normal aerobic growth, partial in vitro fixation and nod+fix- phenotype had much lowered contents of cytochromes aa3 and c, a trace of CO reactive o and P-420. Ets7, capable of aerobic growth but not in planta fixation had only traces of amounts of aa3, but elevated cytochrome c content (compared to Ets1). The CO-difference
### Table 8.5. Relative cytochrome concentration of putative Ets mutants in aerobically grown cells

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Reduced minus oxidised</th>
<th>CO-difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aa3</td>
<td>b</td>
</tr>
<tr>
<td>ANU289</td>
<td>230</td>
<td>690</td>
</tr>
<tr>
<td>Ets 1</td>
<td>+</td>
<td>140</td>
</tr>
<tr>
<td>Ets 3</td>
<td>220</td>
<td>880</td>
</tr>
<tr>
<td>Ets 5</td>
<td>120</td>
<td>360</td>
</tr>
<tr>
<td>Ets 6</td>
<td>110</td>
<td>850</td>
</tr>
<tr>
<td>Ets 7</td>
<td>+</td>
<td>510</td>
</tr>
<tr>
<td>Ets 8</td>
<td>480</td>
<td>1340</td>
</tr>
<tr>
<td>Ets 12</td>
<td>90</td>
<td>360</td>
</tr>
<tr>
<td>Ets 13</td>
<td>250</td>
<td>650</td>
</tr>
</tbody>
</table>

The wild type and mutants were grown in TY medium (Chapter 2). The method of cytochrome spectra analysis is previously described. Quantification of some CO-reactive pigments was difficult for instance, lack of distinction between cytochrome o and cytochrome p-420, hence the relative amounts are presented. + and ++, relative abundance, +, trace, -, not detectable.
spectra showed a dominating c552 and a P-420 (not found in ANU289 and present in trace quantity in Ets1). Thus in these two mutants, little or no nitrogen fixation may be due to a drastic reduction in amounts of cytochrome aa3, c and a slight reduction of CO reactive o. No observable effect on growth of these strains may be because of the presence of cytochrome o and also cytochrome P-420 which appeared in Ets7. Ets8, on the other hand, was characterised by aerobic growth, and wild type nitrogen fixation patterns, and showed inexplicably elevated contents of all cytochromes compared to wild type. Since there was a considerable time interval between secondary screening and spectral analysis, some change in the lesion(s) may have occurred (for example, a reversion in a regulatory gene) during this period, to cause Ets8 phenotype.

In contrast, all transposon-induced mutants (Ets3, Ets5, Ets6, Ets12 and Ets13) exhibited only partial reduction in either a and/or c. The amounts of CO-reactive components particularly of cytochrome o varied among these mutants and in certain mutants there appeared a P-420 probably compensating for the reduction in cytochromes o and/or aa3 content. These mutants were either nod+fix+ or nod+fix−. Thus, transposons which induce single random mutations compared to NTG, were not capable of producing absolute 'nadi' mutants (e.g. Ets1). The findings from these mutant studies are in many respects similar to those observed in R. japonicum (El Mokadem and Keister, 1982).

8.7 Hemoglobins in situ and Parasponia induced by strain ANU289

Leghemoglobin is believed to facilitate (Bergersen et al. 1973, Wittenberg et al. 1974) and stabilize (Stokes 1975) the supply of oxygen to the bacteroid surface, thus allowing the operation of high efficiency oxidative phosphorylation (Appleby et al. 1975).
Relatively little is known regarding such oxygen carrier systems in non-legumes. Davenport (1960) reported the presence of a hemoglobin-like compound in actinorhizal nodules that might be analogous to leghemoglobin, but this needed further confirmation. Later peroxidase activity was demonstrated in the root nodules of four species of nonlegumes but no hemoglobin could be detected in these species (Moore 1964). Wittenberg et al. (1974) reported a range of hemoglobins and non-heme O₂ carrier proteins that could substitute partially for leghemoglobins in the stimulation of nitrogen fixation. Polyphenol oxidase, tyrosinase or peroxidase, capable of reversible oxygenation were proposed as an alternative oxygen carrier systems (Jolley et al. 1974). Due to the presence of an active polyphenol oxidase system, Coventry et al. (1976) postulated that oxy-polyphenol oxidase could act as an oxygen carrier in the Parasponia nodule. However, the possibility of any such alternative oxygen carrier systems in non-legumes was recently excluded because of lack of correlation between the activity of these enzymes and nitrogenase activity (Wheeler et al. 1979).

Studies from the detached nodules (Section 7.5) suggested the presence of an O₂ buffering system in the Parasponia nodule but it was not as efficient as leghemoglobin. Investigations of cytochromes and oxidases in strain ANU289 bacteroids from Parasponia and siratro indicated a general role for these oxidases, but none specific for Parasponia (Section 8.5 and 8.6). It was, therefore, apparent that Parasponia nodules may have a hemoglobin-like compound which is different from that of legumes. In this section attempts are described to isolate and compare the O₂ carrier proteins in both siratro and Parasponia nodules.
8.7.1 Isolation, partial purification and characterization of siratro leghemoglobin

The crude supernatant from the nodule homogenate obtained during bacteroid isolation was saved and later used for leghemoglobin analysis. This supernatant was partially purified using standard laboratory procedures (see Section 2.4.4). The elution profile of siratro leghemoglobin using a linear gradient of acetate buffer (pH 5.6) is shown in Fig 8.7. The profile exhibited one major and three minor peaks. The absorption spectra of one of these fractions (Fig. 8.8) were essentially the same as those of soybean leghemoglobins (Appleby 1974). The relationship between various forms was not further investigated. Since chromatography had provided an incomplete separation of these hemoglobins, disc electrophoresis in polyacrylamide gels was used to separate these fractions further (data not shown). Fraction 56 separated into two bands on these native gels, one major and one minor. The other fractions showed only one major band each. SDS-PAGE electrophoresis of 50 µl of each of the component fractions indicated a molecular weight of about 15,000 (Fig 8.9A). Elution of protein from such gels (Sue Newton pers. comm.) and subsequent N-terminal aminoacid analysis and comparison with other leghemoglobins indicated that siratro leghemoglobin was closely related to kidney bean and soybean c1 leghemoglobin (data for the first 35N-terminal amino acids only).

8.7.2 A hemoglobin-like compound in root nodules of Parasponia

Grinding of Parasponia nodules with the extraction buffer used for siratro and Parasponia bacteroid isolation produced a light green colour supernatant. This supernatant did not contain any detectable hemoglobin-like compound as indicated by its spectral characteristics. In attempts to concentrate the supernatant, it was fractionated with (NH₄)₂SO₄
Figure 8.7. The elution profile of siratro leghemoglobin from DEAE-cellulose (acetate). The method of leghemoglobin extraction and partial fractionation is described in Section 2.4.4. Four major fractions (56, 62, 68 and 82) were obtained as measured by (i) OD at 546nm and (ii) by absorption spectra (see Fig. 8.8).
Figure 8.8 Absorption spectra of partially purified siratro leghemoglobin fraction 62, prepared as described in the text. ———, leghemoglobin without any addition (a similar spectrum is obtained by oxidising with potassium hexacyanoferrate); ———, leghemoglobin reduced with (deoxygenated) 100 µmoles of sodium dithionite and ————, leghemoglobin equilibrated with CO. The spectra was taken with 65 µmoles of leghemoglobin in 25mM phosphate buffer (pH 6.8).
at pH 6.8 between 45 and 80% saturation. The redissolved precipitate was passed through a Sephadex G-25 column equilibrated with 0.1M phosphate buffer (pH 6.8). An aliquot (50 µl) of the elute were run in a SDS-PAGE gel and produced no detectable band in contrast to marker proteins and siratro leghemoglobin fractions (data not shown).

When the grinding buffer was modified such that the new buffer contained a 5mM sodium ascorbate, 2mM EDTA in phosphate buffer pH 6.8 (all other components remaining same) and semi-aerobic conditions were used, a hemoglobin like spectrum was obtained. The optical properties were more like hemoglobin rather than an oxidase or peroxidase, because of a reversible combination with oxygen (see Fig. 8.8). After concentrating 10 fold (a PM10 diaflow membrane, 6 hrs) a 50µl sample was run on SDS-PAGE disc gels (Fig. 8.9B) to determine the molecular weight. Siratro supernatant (25µl and partially purified fraction 62 (40 µl) were run as controls. As shown in Fig. 8.9B, faint bands of differential intensity from the Parasponia crude extract concentrate were seen. One of these bands ran parallel to siratro leghemoglobin and the molecular weight marker protein lysozyme (MW 14,300). Some other major and minor bands were observed on the gel but none corresponded to 36000 (the molecular weight of peroxidase); these bands may be Parasponia nodulins. The concentration of hemoglobin was very low. This may be due to degradation of the protein by tannin formation, during semiaerobic preparation. Likewise a molecular weight of about 15,000 observed in the aerobic isolation may also be a result of degradation during aerobic manipulations. This is not unexpected as Coventry et al. 1976 noted the presence of several other compounds in Parasponia nodules, which interfere in extraction of many soluble plant proteins (Loomis and Battaile 1966). However, such preparations in the initial study was part of the cytochrome study-protocol and was essential from a comparison point of view.
Figure 8.9. The SDS-PAGE electrophoretograms of leghemoglobins.

(A) electrophoretogram of fractions from DEAE-Cellulose (acetate) column. The band corresponding to MW 15000 is of leghemoglobin. Lanes: (a) molecular weight markers (as described in Section 2.4.5), (b) crude extract, (c) fraction 56, (d) fraction 62, (e) fraction 68 and (f) fraction 82.

(B) electrophoretogram of *Parasponia* hemoglobin (a) molecular weight marker (b) fraction 62 from siratro as control, (c) and (d) *Parasponia* hemoglobin crude extract, prepared as described in the text.
More recently, using strict anaerobic isolation procedures, using techniques as outlined above, it was possible to detect a hemoglobin preparation of apparent molecular weight of about 17,000 from *P. rigida* nodules infected with ANU289 (data not shown). Currently this protein is being sequenced to permit further analysis of cDNA clones derived from *Parasponia* nodules (S. Newton pers. comm.).

These preliminary studies being more the by product of work into the other hemoproteins, indicated that a soluble hemoglobin was present in *Parasponia* nodules. Unlike in siratro, an anaerobic technique was essential for *Parasponia* hemoglobin extraction.

A soluble hemoglobin-like protein has now been described in *Parasponia andersonii* nodule extracts (Appleby et al. 1983). This protein present at about 1 mg/g fresh weight was a dimeric protein with an apparent polypeptide molecular weight of about 21,000.

8.8 *Summary*

Studies in this chapter aimed at a further delineation of the oxygen effects on nitrogenase using analysis of symbiotic systems. However, instead of directly involving oxygen, factors which mediate oxygen utilization in both free-living rhizobia (e.g. electron transport chain) and in bacteroids (electron transport chain as well as hemoglobin) were investigated. Several components of the electron transport chain (particularly oxidases) and the hemoglobins are known to play a definite role in the correct usage of oxygen, and consequently produce energy and reductants to 'drive' nitrogen fixation. Moreover, the report of lack of classical leghemoglobins in *Parasponia* nodules at the outset of this work warranted these studies.
Cultured cells of strain ANU289 had a characteristic cytochrome composition representative of *Rhizobium*. Strain ANU289 was similar in this respect to strains 32H1 and CB756 in terms of cytochromes aa₃ and o content. There were some differences in respect of CO reactive cytochromes c₅₅₂ (present in ANU289) and P-420 (absent in ANU289); the situation was the reverse in strains 32H1 and CB756. The electron transport components were also compared in bacteroids from siratro and *Parasponia* nodules. Bacteroids from both host types appeared to have common electron transport components. For example, both types lacked cytochrome P-450 and yielded more or less similar amounts of cytochromes b and c. The differences between these bacteroid types were in terms of cytochrome aa₃, and o and P-420. Distribution of cytochromes in individual bacterial fractions after a discontinuous sucrose gradient fractionation was similar to total bacteroid preparations except for cytochrome aa₃. This cytochrome disappeared in matured bacteroid fractions and was present in increased amounts in the transforming and vegetative fractions, respectively.

The role of these cytochromes/oxidases in nitrogen fixation was determined by isolating mutants defective in oxidase activity. These mutants were characterised for their growth and nitrogen-fixing abilities both *in vitro* and *in planta*. Mutants which lacked one or more of the cytochromes such as aa₃, P-420 and c were capable of nitrogenase expression but the total activity was reduced several fold. Similarly all the mutants were able to nodulate siratro but were either ineffective or partially effective. This indicated a function for these cytochromes not only in symbiotic nitrogen fixation, but also *in vitro* derepression.

Finally, the work on cytochromes and oxidases gave some insight into the hemoglobins in siratro and *Parasponia rigida*. The siratro leghemoglobin was partially purified; the spectra of this purified
fraction appeared similar to that of soybeans. The molecular weight as determined by DSD-PAGE, was about 15,000. Using techniques used for siratro, it was not possible to identify any hemoglobin in Parasponia nodules. However, after modifying the extraction buffer, or using a strict anaerobic techniques, a hemoprotein in the supernatant was observed with typical hemoglobin-like optical properties. An SDS-PAGE gel with both siratro and Parasponia hemoglobin samples showed a band in Parasponia in parallel with siratro leghemoglobin. It is likely that this is a degraded product of the soluble hemoglobin described by Appleby et al. (1983). The low yield and degradation may be due to aerobic techniques used for extraction. Thus, Parasponia contains a soluble hemoglobin, which is perhaps functionally different from that of siratro. The presence of such a hemoglobin in a non-legume is highly significant and may lead to an understanding of the evolution and phylogeny of hemoglobins in angiosperms. Analyses of this nature was outside the scope of this thesis.
CHAPTER-9

GENERAL DISCUSSION

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9. GENERAL DISCUSSION

9.1 Analysis of derepressability - the need for a genetic approach

The phenomenon of in vitro derepression of nitrogenase activity is amazingly complex and poorly understood. This is because several parameters can influence the observable nitrogenase activity. These fall into three major categories: (i) those which affect the amount of enzyme present (i.e. via transcriptional or translational control), (ii) those which affect the enzyme directly by allosteric or conformational control and (iii) those which alter the other requirements for the maintenance of activity such as electrons or ATP. In cultured cells it is difficult to differentiate between some of these effects especially when pleiotropy is considered or when some factors can have effects at two different levels (e.g. oxygen).

An elegant way of understanding the regulation of nitrogenase synthesis is to utilize the gene fusion technique whereby the control regions of one operon are linked to the structural genes of another functionally unrelated operon. For example, K. pneumoniae nif regulation was investigated by fusing the regulatory regions of the nif operon(s) to the E. coli lacZ gene coding for beta-galactosidase (Dixon et al. 1980). With the recent knowledge of nif operons in Rhizobium (Scott et al. 1982, 1983a, 1983b, Hennecke 1983, Carlson et al. 1983), such work will soon be possible in Rhizobium as well.

Studies, to date, of the derepression of nitrogenase activity in free-living rhizobia have mainly focused on nutritional and oxygen requirements. The former was reported to differ from strain to strain (Pankhurst 1981). Why many Rhizobium strains, particularly those belonging to the fast-growing Rhizobium species are recalcitrant to derepression of nitrogenase activity remains a 'mystery' and a matter of speculation. It is most likely that the process is subjected to both physiological and genetical control. No attempt has been made to dissect the phenomenon of derepression using a classical genetic approach.
In the present investigation mutants were produced to make an analysis of three major controlling pathways including carbon metabolism (e.g. EPS synthesis and succinate metabolism), nitrogen metabolism (e.g. ammonium and glutamine assimilation) and energy metabolism (e.g. hydrogen uptake and electron transport system). Although the biochemical nature of these mutants remains unknown at present, this study aimed at investigating the effect of these mutations on the ability to derepress nitrogenase both in vitro and in planta. A definite role of these pathways in derepression of nitrogenase activity was established.

Two mutagens namely transposon Tn5 and NTG were used in this study. Transposon Tn5 is an ideal mutagen since it causes single, random mutations by insertional inactivation. The presence of Tn5 is easily detected through its drug-resistance marker (kanamycin or neomycin resistance) or by DNA sequence homology (see Scott et al. 1982). Mutants isolated here were not scanned for the presence of Tn5, as the kanamycin resistance and their mutant phenotype remained stable throughout the study. The approach as stated in Chapter 1 was to use these mutants and their revertants to see what functions are paramount for efficient nitrogen fixation in the wide range of physiological niches, such as the siratro or Parasponia nodule, or vegetative cultures.

9.2 Techniques and basic requirements for derepression, and the concept of optimisation

Although derepression studies were originally initiated on agar slopes, the liquid culture techniques (both stationary and rapidly agitated) were mostly used. Each of these three techniques had its own experimental advantages. The agar slopes, although unsuitable for physiological studies allowed rapid screening of nif- mutants (Cen et
al. 1982), whereas stationary liquid cultures provided a simple procedure as a source of derepressed cells for studies such as nif mRNA isolation, and rapidly agitated liquid cultures were suitable for physiological studies.

Conditions were defined in this study to derepress nitrogenase using all these techniques. Total nitrogenase activities and specific rates were dependent upon the particular methodology. Highest specific rates were obtained with rapidly agitated cultures. However, for extensive nutritional requirement or mutant studies, such as testing the effects of 15 different carbon sources, the stationary liquid culture was a good compromise between agar slopes and rapidly agitated liquid cultures. Key findings were retested in rapidly agitated liquid cultures. For example, the oxygen sensitivity of ANU289 and 32H1 appeared slightly different in stationary culture but no difference in this regard was confirmed in rapidly agitated culture (see Chapter 7).

The findings reported in Chapter 3 clearly established that strain ANU289 had three basic requirements for the derepression of nitrogenase activity. These were a fixed nitrogen source, one or more utilizable carbon source(s) and most importantly a low O2 tension particularly in stationary (5-10%) and rapidly agitated (0.2-0.25%) liquid cultures (Chapter 3). Thus, in this respect ANU289 was very similar to many other derepressable Rhizobium strains. The only definite exception is the stem nodulating rhizobia from Sesbania rostrata, which can grow on dinitrogen as a sole nitrogen source (Dreyfus et al. 1983). Recently, R. japonicum mutants were isolates, capable of nitrogen fixation in nitrogen-free medium (Nandi and Sen 1981), but these results should be treated with caution, since one out of six isolates was shown to be capable of nodulating soybean. Many other factors namely correct pH, growth stage, cell density, had an important role in producing optimal activity in liquid cultures.
Nitrogenase activity can be readily derepressed in strain ANU289 using the conditions described in Chapter 3. As work proceeded it was possible to optimise the activity (see Chapters 3 and 4). Thus the techniques used currently give uniform quantitative results. Because of this, it is realised that sometimes (especially in the earlier studies) relatively suboptimal conditions were used. However, since proper internal controls were used, even if based on slightly different media formulations, these results are still valid.

9.3 A necessity for nutritional requirement studies

In view of promiscuity of strain ANU289 in nodulating effectively a range of legumes and Parasponia and the finding of Coventry et al. (1976) that Parasponia nodules lack leghemoglobin, an initial hypothesis was that the success of the Parasponia endosymbiont may be due to its intrinsic metabolic patterns. A study of the nutritional parameters controlling nitrogenase activity in strain ANU289 was also warranted because of reported strain- and methodology-dependence of these requirements among other rhizobia. Investigations in Chapter 4 dealt with a wide spectrum of requirements for further optimisation of nitrogenase activity in respect of carbon sources (sugars and organic acids), nitrogen sources, iron, molybdenum and cyclic nucleotides. Indeed, results demonstrated that, in fact, strain ANU289 was different from other cowpea strains in some respects.

Utilization of various carbon sources has been used in the subdivision of Rhizobium into species (Graham 1964). All species of this genus have been shown to possess the Entner-Doudoroff (ED) pathway (Martinez-DeDreys and Arias 1972, Ronson and Primrose 1979), whereas evidence for the presence of Embden-Meyerhof-Parnas (EMP) pathway is contradictory (Mulongoy and Elkan 1977). The pentose phosphate (PP)
pathway has been demonstrated in fast growing rhizobia (Ronson and Primrose 1979) but not yet in slow-growing rhizobia (Stowers and Elkan 1983). In addition, there exists an operational tricarboxylic acid (TCA) cycle in rhizobia (Mulongoy and Elkan 1977, Ronson and Primrose 1979). Although no detailed studies have been made of the various metabolic pathways which operate in strain ANU289, growth on various sugars and organic acids (Table 4.1) indicates the presence of these pathways. Lack of growth on sucrose alone is probably due to the absence of invertase, perhaps a characteristic of slow-growing rhizobia.

Of interest was the observation that strain ANU289 produced higher nitrogenase activity in media supplemented with either hexose sugars, disaccharides or sugar alcohols than with pentoses, which were the most favourable carbon sources for nitrogenase activity in other cowpea rhizobia (Gibson et al. 1976, Pankhurst 1981). Similar to agar cultures (Pankhurst 1981), in stationary liquid a favourable combination of a sugar and a TCA cycle intermediate was necessary. The reason for the 'synergism' between a sugar and a TCA cycle intermediate remains obscure. One could speculate that this may be due to either balancing of pH (Ronson et al. 1981, see Section 4.3.3) or modulation of uptake and/or metabolism of one by the other (McAllister and Lepo 1983). Among several organic acids tested, a few namely succinate, pyruvate or fumarate supported optimal nitrogenase activity. Succinate appeared to play a major role in cellular metabolism and was chosen for further investigations (Section 9.4).

Similar to carbon sources, various nitrogenous compounds affected nitrogenase activity. A favourable reduced nitrogen source was essential in the medium for optimal derepression. In common with many Rhizobium strains only three nitrogen sources, i.e. casamino acids, glutamate and proline supported high nitrogenase activity. Liberation of ammonia into
the surrounding media was another feature of strain ANU289, common to other 'cowpea rhizobia'. In the absence of $^{15}$N incorporation, this conclusively demonstrated nitrogen fixation in strain ANU289. Furthermore, strain ANU289 was different from other cowpea strains in respect of the effect of ammonium and glutamine and this is discussed in a later section (Section 9.5).

Iron and molybdenum are believed to be supplied by the plant for bacteroid nitrogenase and heme synthesis (Cutting and Schulman 1972). In in vitro conditions, unlike the carbon and nitrogen sources, the effects of iron and molybdenum were less pronounced. Although molybdenum was required for nitrogenase activity, similar to Pankhurst (1981), in this study it was difficult to show an absolute need for supplementation. This may be caused by carryover in the inoculant cells and/or low levels of chemical contamination of most media components. The iron requirement for nitrogenase activity was more pronounced, perhaps indicating the more general involvement of iron in the cellular metabolism.

Differential effects of the adenine nucleotides on nitrogenase activity were also observed. It is not known whether these differences resulted from internal metabolism or was caused by uptake differences. For a mechanistic understanding, further investigations of the effect of cAMP are warranted as has been done to demonstrate cGMP effects (Scott et al. 1979, Jones et al. 1981, Van den Bos pers. comm.). Of significance probably is the fact that in strain ANU289, the cAMP effect observed was in contrast to that of strain 32H1 (Pankhurst 1981). Thus the cAMP effects observed are comparable with the carbon source effects on nitrogenase activity of strains 32H1 and ANU289.

In summary, strain ANU289 showed a different spectrum of requirements for derepression of nitrogenase, when compared with other cowpea strains such as 32H1 or CB756. Strains 32H1 and CB756 form
ineffective nodules on *Parasponia*. It is possible that strain ANU289 is highly successful in *Parasponia*, because the plant supplies the infection thread-confined bacteria with the appropriate spectrum of carbon compounds such as sugars and carboxylic acids required for nitrogenase derepression. These compounds are not suitable for nitrogenase expression in other strains (e.g. 32H1 and CB756) and may thus be responsible for fix- phenotype.

9.4 A role for carbon and energy metabolism in nitrogenase activity

The differential influence of different carbon sources on growth and nitrogenase activity in this study was of interest. Because of a relationship between carbon metabolism and oxygen consumption (Agarwal and Keister 1982), it was presumed that carbon sources may exercise their effect by their ability to participate in energy production, or by influencing *in situ* O$_2$ concentration. In the absence of general insight into the physiology of carbon metabolism particularly in slow-growing rhizobia, a role of individual carbon source remains obscure. Studies in Chapter 5 investigated two different aspects of carbon-metabolism: one dealing with intermediary metabolism such as EPS synthesis and the other focusing on succinate metabolism. The role of hydrogen metabolism, representing an interphase between carbon and energy metabolism was also studied.

A negative correlation between EPS synthesis and nitrogenase activity was established in strain ANU289 using the isolated EPS producing mutant and the isogenic mucoid variant (strain ANU288). In strain ANU289 this was tested on combinations of sugars and organic acids. It was possible to conclude that the carbon source, which promoted more EPS synthesis inhibited nitrogenase activity. These observations are in
agreement with observations of Agarwal and Keister (1982), who used 20 different strains and two carbon sources and showed a negative correlation at a physiological level between EPS synthesis and nitrogenase activity.

EPS synthesis is regulated by the carbon source. This regulation may involve the uptake system and/or the existence of an enzymatic machinery for metabolism and synthesis. Little is known about these systems in slow-growers, although in fast-growing rhizobia, transport of carbon compounds is an active process and oxidation patterns could be either inducible, semi-inducible, semi-constitutive or constitutive (Dilworth and Glenn 1981). Additionally EPS synthesis may be controlled at the level of intermediary metabolism and/or polymer synthesis. These processes rely on the energy pool of the cell and may be homeostatically controlled.

The other parameter studied in conjunction with EPS synthesis and nitrogenase activity was oxygen consumption. Previously, using 36 different R. japonicum strains under the same conditions (Agarwal and Keister 1982) found a negative correlation between oxygen consumption and nitrogenase activity. In this study, with strain ANU289 but using 20 different carbon sources, this correlation between these two parameters no longer holds. For example, sucrose which consistently stimulated oxygen consumption in combination with succinate, always supported good nitrogenase activity. It is not known why certain carbon sources stimulate oxygen consumption, while others did not.

In summary, a carbon source may regulate by derepression of nitrogenase synthesis as well as by supporting the enzyme activity by a balanced supply of ATP and electrons. Carbon source may modulate the carbon-nitrogen balance of the cell and thus control the nitrogen status. The latter regulates the derepression of nitrogenase at genetic levels.
(perhaps through not yet demonstrated ntr [nitrogen regulation] genes that have been identified in Klebsiella but not in Rhizobium (Merrick 1982)). Once nitrogenase is synthesized, the nitrogenase activity is limited by the energy derived from carbon assimilation. There exist two possibilities as to how EPS synthesis may affect the energy supply for nitrogenase reaction. Firstly, since these studies were done entirely in stationary liquid medium, the production of varying amounts of slime may differentially amplify the oxygen gradient in the liquid 'cushion'. Thus oxygen utilization and energy availability may directly influence nitrogenase activity. A reciprocal correlation between EPS synthesis and nitrogenase activity was observed also in rapidly agitated liquid cultures where almost all the cells are exposed to uniform microaerobic condition (Agarwal and Keister 1982). It was suggested that the cell wall bound (as is seen in most slow-growing rhizobia) EPS may have reduced the efficient oxygen utilization and thus interfered in nitrogenase activity.

Secondly, under microaerobic conditions nitrogenase and polysaccharide synthesis may compete for energy as was proposed by Agarwal and Keister (1982). Thus the strains which produce large amounts of EPS, the energy is utilised in the production of EPS rather than in the synthesis and maintenance of new proteins such as nitrogenase. The results of the present study are in agreement with this model. However, because of the lack of absolute correlation (-0.65, see Fig. 5.4), the present study recognises that though EPS synthesis is a major competitor for energy, other factors (such as growth) may also be involved. Thus, carbon sources which support rapid growth and hence more EPS do not stimulate nitrogenase activity. Those which allow consistent but slow growth produce low EPS, but due to the balanced supply of ATP and electrons, show maximal nitrogenase activity (e.g. mannitol). This is schematically presented in Fig. 9.1.
Mutants isolated defective in succinate uptake or metabolism provided evidence for a role of succinate in nitrogenase derepression in both the free-living state and in legume nodules. The availability of these mutants and their revertants demonstrated the participation of carbon sources other than succinate in nitrogenase derepression at least in *in vitro* conditions. Although succinate has long been reported to participate in the regulation of nitrogen fixation, the majority of studies have been confined to fast growing rhizobia.

Succinate may have dual functions: a TCA-cycle related function and obviously a function in oxidative phosphorylation. The backward operation of the TCA cycle may generate carbon skeletons (for example α-ketoglutarate) mainly for aminoacid synthesis (Evans 1966). This calls for a co-ordinated operation between nitrogenase, which produces ammonia, and the TCA cycle, which participates in the synthesis of acceptors for ammonia.

A number of mutants unable to grow on succinate (defective in succinate uptake and coupled oxidative phosphorylation) were isolated. Their analysis with regard to H⁺/o ratios, growth on anaerobic conditions, ATPase activity and succinate uptake may be useful in delineating a precise relationship between succinate metabolism and nitrogen fixation.

Uptake hydrogenase activity is distinct from carbon metabolism in the nodule. However, similar to the metabolism of sugars and organic acids, this enzyme system is tightly involved in the energetics of nitrogen fixation. Due to the commercial value of soybeans, the majority of research work on uptake hydrogenase has centered on *R. japonicum* both on bacteroids and on free-living forms. The direct evidence in favour of positive involvement of *Hup* in nitrogen fixation was recently reported (Lepo et al. 1981) by isolating a revertible *Hup*-mutant.
Isolation and analysis of Hup\(^-\) mutants and revertants reported here in strain ANU289 further supports the role of uptake hydrogenase not only in the legume-and nonlegume-Rhizobium symbiosis but also during in vitro nitrogenase derepression. As a part of the screening procedure the putative TTC negative mutants were tested for nodulation in siratro and both Hup and nitrogen fixation activities were measured. The conclusions reached here are in agreement with the previous findings (Lepo et al. 1981), and thus establish the importance of uptake hydrogenase systems in nitrogen fixation in another slow-growing Rhizobium. Since the mutants were produced by Tn5 mutagenesis, it may be possible to isolate and characterise the Hup gene(s) in Rhizobium strain ANU289 using recombinant DNA approaches as outlined by Scott et al. (1982).

Strains ANU288 and ANU289 were Hup\(^+\). It was also shown that observable in vitro nitrogenase activity was influenced by the uptake hydrogenase phenotype. Though such analysis has not been extended to other strains, it appears that there may be a positive correlation between the presence of uptake hydrogenase activity (Hup\(^+\)) and the derepressability of nitrogenase activity in vitro. This idea is supported by the following arguments: (1) both enzymes are derepressed only under microaerobic conditions, (2) most of the slow-growing strains examined for Hup activity are Hup\(^+\), whereas most of the fast-growing strains examined are Hup\(^-\), and (3) an attempt to catalogue some of the Rhizobium strains analysed for both uptake hydrogenase and nitrogenase activity in vitro showed that the majority (about 80\%) of these strains were either Hup\(^+\) Nif\(^+\) or Hup\(^-\) Nif\(^-\). The few discrepancies can be explained on the basis of differential nutritional requirements for the expression of these enzymes. However, a more comprehensive analysis using a larger number of strains is necessary.
It is not unlikely that being Hup+ provides strain ANU289 a special ability to be effective in a range of legumes but it may play a role in effective nitrogen fixation by the non-legume Parasponia (see Section 9.8). Furthermore, stimulation of nitrogenase activity in wild-type by the addition of hydrogen gas in the gas phase suggests the possibility of 'chemolithotrophy' by strain ANU289. This has been found in some other R. japonicum strains (Evans et al. 1982), and could work as a 'survival' mechanism for strain ANU289 in the rhizosphere of Parasponia.

9.5 Effect of nitrogenous compounds on nitrogenase activity

In contrast to carbon and energy metabolism, nitrogen metabolism in relation to nitrogenase activity in rhizobia has drawn more attention in recent years. The highly derepressable Rhizobium strains, namely 32H1 and 61A76 have been particularly helpful in these analyses. The differential effects of various nitrogenous compounds on nitrogenase activity in strain ANU289 (Chapter 4) indicated some similarities of this strain with the abovementioned strains (see Section 9.3). Some potential differences were also apparent.

Although only three compounds, namely glutamate, proline, casamino acids, supported nitrogenase activity (Chapter 4, also see Section 9.3), strain ANU289 was capable of utilizing a range of nitrogenous compounds for aerobic growth on either glucose or arabinose. This suggested that it was not only the ability to support growth but also to simultaneously signal the 'nitrogen control' of the cell of the nitrogen starvation was important. Recently an active uptake system for methylammonium was reported in microaerobic conditions but not in aerobic conditions in
strain 32H1 (Gober and Kashket 1983). Strain ANU289, unable to grow on methylamine, may be similar to 32H1 in this respect. These strains appear to be different from the fast-growing strains, some of which can utilise methylamine for growth (e.g. R. trifolii, Weinman 1981) and in R. leguminosarum methylamine uptake was active and remained unaltered in any of the growth conditions tested (Dilworth and Glenn 1982).

The growth of strain ANU289 on ammonium, coupled with preliminary analysis of assimilatory enzymes (Howitt pers. comm.) indicated that GS-GOGAT is the pathway of ammonium assimilation in strain ANU289. In accordance with the requirement in other 'cowpea strains', strain ANU289 required a source of fixed nitrogen for development of nitrogenase activity. This nitrogen source may permit initial growth, so that the appropriate balance between oxygen concentration and cell number is reached, resulting in nitrogenase synthesis in some cells. This characteristic, coupled with liberation of NH4+ to the surrounding medium indicates that these rhizobia are different in nitrogen control from free-living nitrogen-fixing (e.g. Klebsiella) and Sesbania-Rhizobium strains (Dreyfus et al. 1983). Ludwig (1983) proposed that free-living nitrogen-fixation by Rhizobium sp. occurs in a cell-state divorced from that of growth. In strain ANU289, growth was observed in nitrogen-fixing cultures (e.g. Table 5.2). Also glutamate concentrations exceeding 3 mM inhibited nitrogenase activity. Thus it is likely that the microaerobic nitrogen-fixing cultures may contain both growing and non-growing cell types. Of these, as was proposed by Ludwig, the non-growing type is involved in fixation and crossfeeds the N-limited cells that remain vegetatively growing.

Strain ANU289 was different from 32H1 or CB756 in respect of sensitivity of nitrogenase derepression to glutamine and NH4+ which
have proven to be good nitrogen sources in the latter strains (Pankhurst 1981). In the present investigation both ammonium and glutamine inhibited nitrogenase activity not only in stationary culture but also in rapidly agitated liquid cultures. It is not known whether the inhibition is due to repression of synthesis or modulation of activity or both. The effect of ammonium requires its assimilation since MSX reverses it, thereby eliminating explanations such as ammonium effects via altered membrane energization or pH effects. Chloramphenicol (protein synthesis inhibitor) had a more pronounced effect than NH$_4^+$. This suggested that ammonium inhibition probably involved some metabolic processing at least through GS. Whether a relationship exists between the adenylation properties of GS and derepression or inhibition of nitrogenase activity in ANU289 is currently in progress (Howitt, pers. comm.).

Glutamine also showed effects similar to ammonium. The present study focused on further studies of glutamine assimilation of strain ANU289. In E. coli, the enzyme glutaminase was postulated to be involved in depleting glutamine under low energy conditions (Hartman 1973). This enzyme switched off major biosynthetic pathways, thereby conserving energy. In this study, a substrate inducible intracellular glutaminase was discovered in cultured cells of strain ANU289 and more significantly even in glutamate-grown cells (glutamate is used as a nitrogen source in derepressing cultures). In addition, the cultured cells appeared to have an uptake system for glutamine. While these data add to the further characterisation of glutamine assimilation in strain ANU289 and suggest glutamine per se may play a role in derepression of nitrogenase activity, the mechanisms of glutamine inhibition yet remain uncertain.

Though the precise biochemical nature of the mutants is not known, from the preliminary growth studies these appeared to be defective in
glutamine assimilation (e.g. glutamine transport, GOGAT or glutaminase activity, see Section 6.5). Surprisingly, these mutants also showed higher nitrogenase activity compared to the parent strain. This suggests that glutamine may exert some specific inhibitory effect.

Thus, in summary, it is likely that glutamine may have some specific effects on nitrogenase activity but further study of these mutants and a study of the relationship between derepression of nitrogenase activity and enzymes involved in ammonium and glutamine assimilation at a biochemical level are necessary before any conclusions regarding the mechanism of inhibition can be drawn.

9.6 Regulation by oxygen

Experiments probing the oxygen requirement and sensitivity of nitrogenase expression by strain ANU289 seemed important in view of the reports such as the absence of leghemoglobin in *Parasponia* nodules (Coventry et al. 1976), and the high content of cytochrome aa3 in *Parasponia* bacteroids (Appleby et al. 1981). It was a distinct possibility that nitrogenase in strain ANU289 inside *Parasponia* nodule is salvaged from O₂ damage by 'respiratory protection'. Because of several commonalities between bacteroids and microaerobic bacteria (Avissar and Gollop 1982), it was reasonable to assume that if nitrogenase in *Parasponia-Rhizobium* strains were more tolerant to oxygen, for instance due to a mechanism such as 'protective respiration', it would be reflected under in vitro conditions. Derepression of nitrogenase activity in this strain provided results quite contrary to this hypothesis. Strain ANU289 had an oxygen requirement similar to other *Rhizobium* strains (e.g. 32H1 and CB756) and was equally sensitive to high oxygen concentrations.
The above experiments clearly demonstrated that some other oxygen protection mechanism may prevail in planta. This is consistent with the recent discovery of a hemoglobin (see Chapter 8 and Appleby et al. 1983) in Parasponia.

Comparison of the oxygen sensitivity of nitrogenase activity in Parasponia and siratro detached nodules yielded significant results. Although some variations were observed in the results because of detached nodule-assay procedures (Bergersen 1970, Pankhurst and Sprent 1976), these could be easily controlled. In the present study the nodules from both plant species were compared under identical conditions, that is with 10% C2H2 in the gas phase and incubated at 22°C. Maximum nitrogenase activity observed at 30 to 90% v/v oxygen in siratro was similar to that found in soybean nodules (at 50-90% O2 v/v) (Bergersen 1970). In contrast, the oxygen sensitivity curve of detached Parasponia nodules, with an optimum at 30% O2 (v/v), was very similar to the detached Casuarina cunninghamiana nodules (Bond 1961). The difference between Parasponia and siratro was clear cut and it was distinct from the small variations sometimes observed between different legume species. A narrow pO2 optimum in Parasponia nodules suggests that the mechanisms to buffer oxygen and protect nitrogenase may be restricted. Perhaps Parasponia hemoglobin (Chapter 8) is different and less efficient in its oxygenation properites compared to siratro hemoglobin. This is probably a functional representation of the recent finding that the off-rate constant (0.3 sec⁻¹ at pH 6.8, 20°C) of Parasponia hemoglobin is one-thirteenth of oxyleghemoglobin (Appleby et al. 1983).
9.7 Role of ANU289-cytochromes in nitrogen fixation by siratro and Parasponia nodules

As stated above, oxygen sensitivity of in vitro nitrogenase derepression was similar in strain ANU289 to other 'cowpea rhizobia' but yet a differential oxygen sensitivity in detached nodules of siratro and Parasponia could be demonstrated. In view of these findings, a comparative study of cytochromes was undertaken. This involved the analysis of cytochromes in cultured cells of strain ANU289, its bacteroids from both Parasponia and siratro nodules and finally electron transport mutants.

The cytochrome spectra of cultured ANU289 cells were typical of Rhizobium in general. But when compared with strains such as 32H1 and CB756, strain ANU289 exhibited slightly different CO reactive pigments. The differences in cytochrome type and contents among these strains may be insignificant or it may be that an ANU289-type cytochrome is essential for effective nitrogen fixation in Parasponia. Similar strain differences were previously seen in R. japonicum. Cytochromes observed in cultured cells were reported to undergo transformation in nitrogen-fixing conditions both in the symbiotic (Appleby 1969 a, b, Keister et al. 1983) as well as in the asymbiotic state (Evans and Christ 1980). Moreover, Evans and Christ (1980) reported a cytochrome P-450 under microaerobic nitrogen-fixing conditions in strain 32H1. Thus the differences observed in cultured cells between ANU289 and 32H1 described in Chapter 8 may be real, in view of the contrasting cytochrome profile observed in nitrogen fixing cells of 32H1 (Evans and Christ 1980) and ANU289 (this study).

The fractionated bacteroids of strain ANU289 from nodules of siratro and Parasponia distributed themselves into three almost equal
fractions. Also consistent with the report of Ching et al. (1977), the cytochrome \textsubscript{aa3} content in these fractions decreased with the nodule age. The matured bacteroids contained minimal or no cytochrome \textsubscript{aa3} and increasing amounts were found in transforming and vegetative bacteroids. In addition, in unfractionated preparations Parasponia bacteroids contained cytochrome \textsubscript{aa3}, in reduced amounts compared to those of siratro and previously reported NGR231-\textit{P. rugosa} (Appleby et al. 1981) bacteroids. In contrast to these studies, Keister et al. (1983) observed 90-99\% of the bacteroids in soybean nodule as matured bacteroids and that the cytochrome \textsubscript{aa3} was consistently present in these bacteroids, although the amount decreased with nodule age.

Both qualitative and quantitative differences in cytochrome \textsubscript{aa3} were observed among strains of \textit{R. japonicum} (Keister, pers. comm.). It is thus apparent that cytochrome \textsubscript{aa3} may be more widely distributed in bacteroids of various species and strains than previously suspected, and this probably reflects the extent of genetic variation among various rhizobia. Because both siratro and Parasponia nodules fixed almost equal amounts of nitrogen, no direct correlation can be determined between nitrogen fixation and the presence of cytochrome \textsubscript{aa3}. These findings with bacteroids coupled with cytochrome \textsubscript{aa3} content observed in microaerobic cultures with 32H1 (Evans and Christ 1980) have disproved the dogma that this cytochrome always appears in microaerobic conditions and/or conditions of abundant heme synthesis conditions (Avissar and Nadler 1978).

The other commonality between Parasponia and siratro bacteroids was the absence of cytochrome P-450 as was the case with NGR231-\textit{P. rugosa} bacteroids (Appleby et al. 1981) and \textit{R. leguminosarum} (Kretovich et al. 1973). In the latter case it was suggested that cytochrome P-450 may be
different and may have undergone transformation manifesting at 420 and 424 nm in the CO difference spectrum of the soluble fraction, similar to that seen in liver microsomes (Omura and Sato 1964). Evidence from experiments with Parasponia showed that such a possibility is unlikely as no P-420 was observed in Parasponia bacteroids.

Strains such as 32H1 (cowpea miscellany) and 61A76 (R. japonicum) have the reputation of being in the group of strains which show high specific rates of nitrogenase activity (200-445 nmoles C2H2 hr⁻¹ mg protein⁻¹) in vitro (Agarwal and Keister 1982). Both of these possess cytochrome P-450 and high cytochrome oxidase activity (Evans and Christ 1980, Keister et al. 1983) in contrast to strain ANU289. It is tempting to speculate that these cytochromes may play a vital role in oxygen utilization so as to maximise ATP production and thus aid the nitrogenase activity. Examination of several strains is required in order to establish a functional relationship between the presence of P-450/aa3 and derepressability.

The dominating CO reactive components in Parasponia bacteroids were cytochromes _c552_ and _o_, whereas siratro bacteroids showed CO reactive _aa3_, _o_ and a possible _P-420_. The increased cytochrome _o_ in Parasponia bacteroids was probably compensating for the loss of cytochrome _aa3_. It was also suggested from the spectra of fractionated bacteroids that a gradual loss of cytochrome _aa3_ was associated with an increase in cytochrome _o_ content. Thus, the overall electron transport system in these bacteroids, particularly from Parasponia, was very typical of cultured ANU289 cells. The presence of these cytochromes may be advantageous to cope with the moderately high external oxygen tension, also characteristic of Parasponia nodules (Tjepkema and Cartica 1982).
Nitrogenase expression in Rhizobium in relation to cellular metabolism. This model is based partly on the results obtained in the present study and other such studies documented in the literature.

Whole cell nitrogenase activity may be controlled at two levels: derepression of nitrogenase synthesis and maintenance of activity. Nitrogenase synthesis may rely on signalling by carbon:nitrogen ratio of the cell (or, 'nitrogen status'). This is dependent upon the uptake and metabolism of exogenously supplied carbon or nitrogen source, particularly under microaerobic conditions. Because of evidence such as (1) only few but common nitrogenous compounds support optimal activity in many Rhizobium strains, (2) no relationship exists between the ability of a nitrogen source to support growth and nitrogenase activity, it is speculated that these compounds play a predominant role in controlling derepression of synthesis. Oxygen has a definite negative effect at this level.

In contrast, nitrogenase activity is regulated predominantly by the rate of carbon metabolism which determines the total ATP and electron pool of the cell. Rapid metabolism results in diversion of ATP and electrons to intermediary metabolism (e.g. EPS synthesis) and/or growth (protein synthesis), less or none being available for nitrogenase reaction. A slow metabolic pattern ensures a balanced supply of these requirements for nitrogenase activity. The efficiency of ATP production for nitrogenase reaction is also increased if uptake hydrogenase is present under these circumstances. Oxygen appears to have a greater inhibitory effect on activity than on synthesis. Presence of appropriate oxidases (e.g. P-450 along with oxidases responsible for protective respiration') coupled with membrane bound uptake hydrogenase may play a role in ensuring sufficient ATP production, while keeping the intracellular oxygen level quite low to prevent inactivation of nitrogenase proteins.
Nitrogen source uptake

Carbon source uptake

Membrane

Energisation

Inside

General metabolism

Nitrogenase

Amino acid metabolism

TCA cycle

Electron donor

Electron acceptor

ATP

ADP + Pi

Hydrogenase

Cytochromes

Respiratory chain

O₂ → 2H⁺

O₂ → ½O₂

NH₄⁺

N₂ H⁺

EPS

H₂O

H₂O

H₂O

NH₄⁺

3GAL

3M

3G

4Me-Gal

Lipid carrier

3G

2e⁻

ED EMP

Inside

Outside

Outside
Jones et al. (1978) showed that the bacteria which had cytochromes $\text{aa}_3$ and $c$ as terminal oxidases and which used cytochrome $c$ as electron donor produced about 6 moles of ATP per mole of oxygen, whereas strains which lacked cytochrome $c$ produced only 4 moles of ATP. In the present study twelve oxidase defective mutants were isolated and some of these were partially characterised in order to define the functions of the cytochromes. These mutants were characterised by normal aerobic growth patterns, reduced in vitro and variable in planta nitrogenase activity as were the oxidase mutants in $R$. japonicum (El Mokadem and Keister 1982). The studies with ANU289 thus further generalize the characteristics of cytochrome oxidase mutants in general and in Rhizobium in particular.

Of the eight mutants (Ets1) which were spectrally characterised, one was almost deficient in cytochrome $\text{aa}_3$ and $c$ and showed drastically reduced in vitro nitrogenase activity and very little symbiotic fixation. Another (Ets7) also lacking cytochrome $\text{aa}_3$ but with elevated cytochrome $c$ content was partially effective. The ability of growth of the mutants lacking one or two oxidases implied multiple oxidation pathways in strain ANU289. Characterisation of in vitro and in planta nitrogenase suggest that cytochromes $\text{aa}_3$ and $c$ are probably the functional terminal oxidases. In the absence of low temperature spectroscopy it was difficult to ascertain the mutants' cytochrome $c$ phenotype, although the cytochrome $b$ content in reduced minus oxidised spectra and CO difference spectra suggested that some of the mutants may lack partially or completely cytochrome $c$. In contrast to the parent strain, some of the mutants showed a soluble $P$-420, suggesting that the loss of some cytochrome(s) was compensated for by other oxidases.
To isolate cytochrome mutants NTG appeared to be a better mutagen than Tn5 presumably due to its ability to cause multiple mutations. This may be essential to obtain absolute oxidase mutants because of the presence of a branched respiratory chain in rhizobia. As indicated in this initial study using NTG, it should be possible to isolate as many mutants as can be biochemically and physiologically analysed. Although it is not difficult to analyse for gross changes in the major hemoproteins in the cell, it must be stressed that the identification of minor changes particularly in view of 'compensatory effects' is very complex to analyse and labour-intensive.

9.8 *Parasponia*-strain ANU289, as a model symbiotic system

There were two major goals of the present study. One was to characterise strain ANU289 both physiologically and genetically for its in vitro nitrogenase derepression ability. Based on the results obtained from studies with strain ANU289 and other such studies documented in the literature, a model for nitrogenase expression in *Rhizobium* in relation to cellular metabolism is presented (Fig. 9.1). The other objective was to understand what is special about strain ANU289, so that it was highly flexible and capable of effective nitrogen fixation in *Parasponia*. Obviously the two objectives are interrelated.

Most of the mutants produced during the course of this study were tested for nodulation on *siratro*. Using the procedure outlined in Chapter 2, some of the mutants representing *Suc*, *Hup*, *Asm* and *Ets* phenotypic classes, were tested for nodulation and nitrogen fixation in *Parasponia*. All mutants tested were capable of initiating nodulation but nitrogen fixation varied. The data are presented in Table 9.1. The results obtained were almost in keeping with that of *siratro*.
Table 9.1 Acetylene reduction by Parasponia nodules containing either strain ANU289 or its mutants

<table>
<thead>
<tr>
<th>inoculant strains</th>
<th>acetylene reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU289</td>
<td>8.3 ± 0.25</td>
</tr>
<tr>
<td>ANU289-H-2</td>
<td>0.7 ± 0.10</td>
</tr>
<tr>
<td>Ets2</td>
<td>3.2 ± 0.35</td>
</tr>
<tr>
<td>Ets4</td>
<td>0.5 ± 0.13</td>
</tr>
<tr>
<td>Ets5</td>
<td>4.1 ± 0.25</td>
</tr>
</tbody>
</table>

Acetylene reduction was measured as µmoles/g FW/hr.

A few observations suggest that the symbiosis of strain ANU289 with Parasponia is as good as or even more efficient than with siratro. The total population of bacteroids per unit nodule mass involved in nitrogen fixation is definitely less (about 50%) in Parasponia compared to siratro. This is evident from (1) ultrastructural studies (Price et al., in preparation) where it was estimated that siratro and Parasponia nodules contained approximately 654 and 295 bacteroids per 1000 cubic microns, respectively; and (2) total mass of bacteroids isolated in identical conditions (see Section 8.5). Yet total nitrogen fixation is almost similar in both nodule types (Chapter 7) and energy efficiency of nitrogen fixation of Parasponia is similar to that in legumes (Tjepkema and Cartica 1982). From the results of the present investigation and coupled with those of others, it is postulated that the amplified effectiveness may be because of two reasons: efficient utilization of carbon sources available and the multiple means of oxygen usage while protecting nitrogenase from oxygen damage.

One of the unique features of Parasponia nodules is that rhizobia do not release from infection threads (equivalent to 'bar' mutant in legumes, Vincent (1980)); however nitrogen fixation takes place in these threads. Since bacterial release occurs when strain ANU289 invades
siratro (where it is essential for nitrogen fixation) but not in Parasponia, the release must be a host-controlled character. Though the precise composition of these threads are not known, the thread wall may be quite similar to legume-infection threads. These threads may contain a complex mixture of carbohydrates including the primary translocate sucrose or even those resulting from thread-wall degradation. Degeneration of thread wall (less electron dense) was consistently observed in more matured 'fixation' threads than in infection threads (Price et al. in preparation).

In vitro studies (Chapter 4) showed that strain ANU289 as compared to strain 32H1 (Pankhurst 1981) more efficiently used hexose sugars/sugar alcohols as joint carbon sources for nitrogenase derepression as compared to 32H1 (Pankhurst 1981). Efficient metabolism of sucrose was suggested from increased oxygen uptake, when sucrose was used in conjunction with succinate (Chapter 5). Ultrastructural studies provided additional evidence as the production of PHB was magnified in Parasponia bacteroids compared to siratro (see Fig. 9.2), both grown under identical conditions. This may be due to the abundance of carbon compounds in view of the degradation for the thread wall and also the reduced mass of bacteroids. Since PHB synthesis is known to serve as an electron and carbon 'sink' at very low dissolved oxygen concentration (Jackson and Dowes 1976), accumulation of PHB probably indicates efficient sugar metabolism resulting in sufficient reducing power and ATP. It is thus necessary to examine other Parasponia isolates for utilization of sucrose under aerobic/microaerobic conditions. Such studies are currently undertaken by G. Bender (pers. comm.). Preliminary results are in favour of the above hypothesis.
Figure 9.2. Electron micrographs of bacteroids of strain ANU289 from (A) *Parasponia* and (B) *siratro* nodules, showing the poly-β-hydroxybutarate (PHB) accumulation. Magnification: x66,000. B = Bacteroid, T = Fixation-thread. These micrographs were obtained by Mr G.D. Price.
Parasponia nodules possess a hemoglobin (Chapter 8), though its function in nitrogen fixation yet remains to be demonstrated. It is apparently different in its oxygen buffering capacity from the leghemoglobin in siratro nodules (Chapter 7). The role of this 'novel' hemoglobin in nitrogen fixation is being studied at present (P.M. Gresshoff pers. comm.) by employing two possible approaches: one involving heme-deficient mutants and the other being studies with isolated bacteroids with or without this hemoglobin.

The free oxygen concentration in the proximity of bacteroid membrane is moderately high (200 nM - 1µM). This is evident from the bacteroid cytochrome composition which was similar to aerobically grown cells (Chapter 8) or R. leguminosarum where free oxygen concentration was about 1 µM, Appleby et al. 1981 and also from oxygen microelectrode studies (estimated to be about 280 nM, Tjepkema and Cartica (1982)). Coincidentally at microaerobic conditions (with about <1µM dissolved oxygen tension) ANU289 cells derepressed in vitro showed 3-5 fold stimulation of oxygen consumption and higher nitrogenase activity (Chapter 5) in the presence of sucrose and succinate. While the cytochrome pattern suggests a higher oxygen concentration outside the bacteroid in both Parasponia and siratro nodules (compared to soybean), abundant deposits of PHB (which accumulates only at low dissolved oxygen, Jackson and Dowes 1976) in Parasponia in contrast to siratro bacteroids indicate lower intracellular oxygen concentration in the former.

Furthermore, strain ANU289 bacteroids are uptake hydrogenase positive. This enzyme was described as an oxygen scavenger (Dixon et al. 1972, Evans et al. 1982). Dadarwal et al. (1982) showed that nodules formed by a Hup⁺ strain but low in leghemoglobin were as efficient or even better than those formed by Hup⁻ strain containing a high level of
leghemoglobin. Thus uptake hydrogenase partially supplemented the $O_2$ regulation function of leghemoglobin. The uptake hydrogenase may play a similar role in oxygen utilization and protection of nitrogenase from oxygen in *Parasponia* nodules.

In essence, the study of some of the factors controlling nitrogenase activity in strain ANU289 has pointed out a few special features of the *Rhizobium-Parasponia* symbioses. It should be emphasized that this study represents the first step towards understanding *Rhizobium*-non-legume symbioses. Clearly several questions remain unanswered. To answer these, a combination of physiological, biochemical and genetical analyses of both symbiotic partners are essential. It is hoped that this study will aid in any future investigation of this kind.
Were I to await perfection, I would never finish this book.
A 13th Century Chinese proverb


Prior to this investigation, studies on derepression of nitrogenase activity in fast-growing Sinorhizobium trifolii strain T1 were undertaken. Some success in derepression was achieved by using a co-culture system, which involved strain T1 and white clover cells. However, due to lack of consistency in results (because of a number of uncontrollable factors), the project was considered unsuitable. Some of these studies are listed below and the publications are included in the appendix.

**APPENDIX**


Prior to this investigation, studies on derepression of nitrogenase activity in fast-growing Rhizobium trifolii strain Tl was undertaken. Some success in derepression was achieved by using a co-culture system, which involved strain Tl and white clover cells. However, due to lack of consistency in results (because of a number of uncontrollable factors), the project was considered unsuitable. Some of these studies are listed below and the publications are included in the appendix.


Nitrogenase function represents the final operative phenotype of the legume root nodule symbiosis. Its synthesis and activity results from the progressive interaction of two epistematically controlled developmental pathways, each partially controlled by either symbionts' genotypes, so that plant cortical cells differentiate into a nodule-producing meristem while concomitantly free-living rhizobia (normally unable to express nitrogenase functions) differentiate to become nitrogen-fixing bacteroids. Just as the early steps of infection and nodule initiation require the molecular interaction between host and rhizobia, so one finds specific deployment of both plant and bacterial functions to establish and control bacteroid nitrogenase activity. An obvious example of this fact is illustrated by the "dual" origins of leghemoglobin components.

Little is known about the regulation of nitrogenase in rhizobia (in contrast to Klebsiella pneumoniae or Azotobacter) mainly because this phenotype is restricted to the symbiotic bacteroid form for most strains and thus involves a complex experimental system plagued by nodule heterogeneity in regards to age, bacterial form and plant fitness. A major question regarding the regulation of nitrogenase expression centres on the mechanism involving either derepression or induction. The first alternative would imply that nitrogenase is repressed during the vegetative phase and that "environmental-nutritional conditions" in the nodule cell inactivate this repression system and thus allow nitrogenase gene expression to occur. Alternatively, an induction process implies that specific inducible molecules, probably legume cell derived, specifically activate the expression of the nitrogenase genes.

Historically, studies of the free-living bacteria such as Clostridium, Klebsiella and Azotobacter were analysed to provide a 'model' system for nitrogenase regulation in the symbiotic state of rhizobia. This reductionist approach was slightly reversed when researchers started to correlate whole-plant physiological properties such as photosynthetic rate or nitrate reduction, with the developmental appearance and activity of symbiotic nitrogenase. These approaches were later refined by the isolation and characterisation of nodule protoplasts and bacteroid preparations (Hoit and Broughton, 1979; Schetter and Hess, 1977). These experimental techniques produced a composite picture showing that nitrogenase activity requires sufficient reductant and ATP (supplied jointly by photosynthesis) and that microsorobic conditions are necessary to ensure nitrogenase stability. In the legume-rhizobia symbiosis this is achieved through the action of leghemoglobin, which supplies the bacteroid with a steady, but low amount of oxygen. Furthermore, all studies indicated that a range of substances such as nitrogenous compounds interfere with nitrogenase activity.

The reductionist approach was extended by attempts to obtain nitrogenase activity of rhizobia in vitro. Initial experiments concentrated on co-culture of rhizobia with legume callus cultures (Holsten, Burns, Hardy and Hebert, 1971) and with non-legume cell cultures (Child and Laane, 1974) which illustrated that some strains of rhizobia fixed nitrogen when cultured in association with plant callus. Furthermore, it was shown that a specific strain of cowpea rhizobia, namely 32H, was capable of acetylene reduction in free-living conditions (Child, 1975; Scowcroft and Gibson, 1975; McCall, Elliott and Dilworth, 1975).

Studies with Rhizobium sp. strain 32H and other "slow-growing" strains (such as C3756, C1376) allowed the elucidation of environmental factors which resulted in the development of a functional nitrogenase system (Gibson, Scowcroft and Pigan, 1977). It is of interest to note that parameters such as low O₂ concentration, the effect of nitrogenous compounds and the supply of carbohydrate, mirrored findings obtained with whole plants, isolated bacteroids or nodules. The work with strain 32H more significantly demonstrated that (a) nitrogenase genes are bacterial, (b) strain differences are profound and (c) conditions similar to the plant environment had to be obtained. The fact that only for selected strains, and these mainly in the slow-growing category, showed nitrogenase activity in, or in defined culture medium, suggested that our understanding of the mechanism of derepression-induction was far from complete.
Table 1. Acetylene Reduction in Primary Induction Experiments

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>R. trifolii strains</th>
<th>Maximal acetylene reduction (in moles C(_2)H(_2) day(^{-1}) vial(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>TI</td>
<td>43</td>
</tr>
<tr>
<td>003</td>
<td>TI</td>
<td>22</td>
</tr>
<tr>
<td>012</td>
<td>TI Sp</td>
<td>6</td>
</tr>
<tr>
<td>013</td>
<td>TI Km</td>
<td>9</td>
</tr>
<tr>
<td>014</td>
<td>TI Km</td>
<td>22.8</td>
</tr>
<tr>
<td>015</td>
<td>TI2</td>
<td>6.7</td>
</tr>
<tr>
<td>Control (no C(_2)H(_2) addition)</td>
<td>For all test strains</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Table 2. Acetylene by R. trifolii strain TI Km with unfractionated* clover PCM

<table>
<thead>
<tr>
<th>Concentration of added PCM</th>
<th>n moles C(_2)H(_2) day(^{-1}) vial(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCM only (0.2 ml)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>PCM (0.2 ml) + cells</td>
<td>473.5</td>
</tr>
<tr>
<td>Medium only</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Medium + cells</td>
<td>0.1</td>
</tr>
<tr>
<td>Medium + PCM (0.1 ml) + cells Day 4</td>
<td>543.4</td>
</tr>
<tr>
<td>Medium + PCM (0.5 ml) + cells Day 6</td>
<td>556.0</td>
</tr>
<tr>
<td>Medium + PCM (0.1 ml) + cells Day 6</td>
<td>171.8</td>
</tr>
<tr>
<td>Medium + PCM (0.5 ml) + cells Day 6</td>
<td>272.4</td>
</tr>
</tbody>
</table>

* The plant supernatant was harvested, filtered, centrifuged (20,000 g, 20 min), lyophilized and filter-sterilized before being used in secondary induction experiments.

Future experiments clearly need to monitor the gas phase of the co-culture to optimize the stability of PCM components. Furthermore, extreme care must be taken when isolating and storing PCM preparations. During secondary induction on media based on FS supplemented with sucinate, the culture should be buffered to prevent an increase in alkalinity. These words of caution are added here to illustrate that not all factors of this experimental system are understood.

However, the clear results obtained have indicated that soybean PCM can cause the induction of nitrogenase activity in a wide range of free-living rhizobia such as R. trifolii, R. leguminosarum, R. meliloti and R. japonicum. Similarly, we have been able to induce nitrogenase activity in R. japonicum using clover PCM. Thus the process of induction is non-specific, i.e. PCM produced from the soybean - R. japonicum or clover - R. trifolii co-cultures is active on other Rhizobium strains. Furthermore, co-culture of soybean with R. trifolii also allows the isolation of active PCM.

The question of the molecular components of PCM remains unanswered. Clearly the plant culture requires the presence of living rhizobia, suggesting a biosynthetic output by the bacteria, to produce PCM. This component(s) is dialysable. The plant cell in return, being cultured in nitrogen-deficient conditions, produces a range of compounds, that affect either the bacteria in the co-culture, or in secondary activation affect the free-living cell. This compound(s) is dialysable as well, can be chromatographed on sephadex and is highly oxygen-sensitive. Preliminary data by Reporter suggest that the soybean PCM component needed for nitrogenase activation co-migrates with copper (Storey and Reporter, 1980). No such correlation for copper has been found for the clover system, although other metals may be involved.

Recently, it was found by Reporter et al. (Reporter, Skotnicki and Rolfe, 1980) that PCM can affect rhizobia by increasing NAP production and stimulating respiration. Whether this effect is coupled to the nitrogenase activity remains unresolved.
The isolation of strains of rhizobia capable of in vitro nitrogen fixation, however, facilitated a range of studies into morphological changes, oxygen gradient formation (Rank-hurst and Craig, 1978), and catalytic enzyme analysis (Kiss, Vincent, Salum, Forrai and Kondorosi, 1979), cytochrome analysis (Appleby, Turner and Macnicol, 1975) and genetic studies involving nitrogenase deficient mutants (Maler and Hill, 1975). However, this trend diverged itself from the plant. The importance of plant cell material was re-emphasised in experiments by Reporter (Reporter, 1975; Bednarzki and Reporter, 1975; Storey, Rainey, Pope and Reporter, 1975), who described factors produced in legume-rhizobia co-cultures that facilitated the induction of nitrogenase activity in a wide range of rhizobia under microaerobic conditions and a nutritional environment similar to those described for 3211. The experimental system used by Reporter and associates involved the culture of soybean suspension cells (either soybean variety 1970 or var) in the presence of membrane-filter or dialysis membrane restrained Rhizobium japonicum strain 3BAS1-18-135, a strain which had failed to show nitrogenase activity in a range of defined media.

The induction medium is based on medium B5 described by Gabor and Evehleigh (Gamborg and Evehleigh, 1961), modified by lowering the concentration of iron chelate (either totally absent or supplied at about 1 ppm iron), and omitting both phytochelatins and potassium nitrate. Plant cells are inoculated at a high density, about 50% of the culture volume (Grosshosh and Boy, 1974) while bacterial cultures are diluted within their compartment to about 5 x 10⁵ cells/ml. Culture proceeds at 28°C with slow agitation (110 rpm) or magnetic stirring (as in Reporter's early experiments) over a period of 5 to 7 days at which time time the bacteria are harvested and tested for acetylene reduction. This procedure is termed Primary Activation. If nitrogenase inducing factors are present within the co-culture as indicated by activity in primary activation experiments, the plant supernantant is collected, filtered, sterilised, and stored under argon until further testing. This test involves the induction of nitrogenase activity in free-living rhizobia grown in the complete absence of plant tissue and is termed Secondary Activation.

The medium utilised for secondary activation comprises nitrate-free B5 salts, 10 ppm iron chelate, vitamins (Grosshoff and Boy, 1974), 20 mg/l potassium succinate, 100 mg/l casein hydrolysate and variable amounts of PCM (unfractionated). Experiments by Bednarzki and Reporter (Bednarzki and Reporter, 1975) and Reporter et al. (Reporter, Chotnicki and Rolf, 1980) have shown that PCM can have inhibitory as well as stimulatory effects. Thus an optimum concentration of added PCM needs to be determined with every experiment. In practical terms this means that any one experiment involves three or four replicates with differing PCM additions. As yet there is no means of predicting the appropriate concentration of PCM to be added to give optimum nitrogenase activity. Furthermore, the variable nature of the co-culture system may sometimes produce PCM preparations which fail to produce activity in primary induction but which show activity at low dilutions in secondary activation. Nitrogenase activity in secondary induction experiments as measured by acetylene reduction (the gas phase above the induction medium is 25% argon, 1% oxygen and 45 acetylene) can be determined after 72-96 hours of static culture at 28°C. Acetylene reduction and hydrogen evolution can be measured and the ratio calculated. Contamination tests involving plate assays, tests for genetic stability of antibiotic markers and modulation tests on recovered bacterial clones indicate that observed activities are due to the inoculant rhizobia. Ethylene production detected in these experiments is dependent on the presence of plant, acetylene and PCM, indicating that nitrogenase activity is being measured.

The observations with the soybean system were tested using white clover cell cultures (Grosshoff, 1980). Induction conditions and secondary activation media used for soybean PCM studies were found to! be critical for the production of clover PCM. Some general conclusions can be drawn from PCM experiments conducted in our laboratory with the white clover - Rhizobium trifolii strain T1 system (Tables 1 and 2). The system as described here is highly variable. PCM preparations show little predictability in terms of induction rates and nitrogenase activity. For example, using the clover system, primary activation rates of 0-11 to 0-61 stethylene day⁻¹ per cell were obtained. Variability was minimal within any one batch of PCM. In the experimental design attention was paid to eliminate as much variability as possible. Cell cultures and bacteria were grown to identical phases of their growth cycle, media were standardised, length, rates of aeration and assay conditions were kept constant. Despite these precautions, nitrogenase activity could not be measured in every secondary activation experiment. This degree of variability at present hinders the advance to elucidate the precise physiological conditions required during the co-culture, harvesting and secondary activation phase. Recent experiments have indicated the extent to which oxygen lability of PCM components involved in nitrogenase induction. Thus prolonged co-culture (normally kept under aerobic conditions) could affect the level of PCM factors.
The future research outlook is (a) to reduce the variability of the experimental system by increased control of gaseous phases; (b) to identify the molecular signals exchanged between plant and bacteria and (c) to utilise these compounds to investigate the mechanism of nitrogenase control in rhizobia.

REFERENCES:

NITROGENASE ACTIVITY IN FREE LIVING FAST GROWING RHIZobia

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INTRODUCTION

Asymbiotic nitrogen fixation (acetylene dependent ethylene production) by fast growing rhizobia have recently been reported using different chemically defined media (1,2,3). However, a reliable technique for inducing nitrogenase activity is still lacking. As an alternative, the chemically defined medium can be substituted or coupled with plant-cell conditioned medium (PCM) produced by legume rhizobium-cell co-cultures (4). Induction of nitrogenase activity in different rhizobial strains using this approach has an important bearing on understanding the molecular basis of symbiosis. The present study reports the success in inducing nitrogenase activity in fast growing R.t trifolii strain Tl and others using this approach.

Materials and Methods: Co-culture: Dialysable plant substances capable of influencing rhizobial nitrogenase activity in vitro were obtained from 5-7 days co-culture of white clover cells in suspension surrounding R.trifolii cells within a dialysis bag (molecular cut-off 50,000) supported from a glass manifold. The white clover (line C2570, root callus derived) and rhizobial cells (strain Tl and it's mutants) were precultured in clover suspension medium (5) and Bergersen's modified medium (6) respectively to logarithmic phase before being placed into co-culture flask. Plant and rhizobial cells (adjusted to a density 3x10 bacteria per ml) were suspended in a modified BS medium (6) devoid of nitrate and hormones containing low iron (0 to 2x10 M) supplied as iron-cbolate. Incubation of co-culture vessels was by gyratory agitation (110 rpm) at 26 C. Rhizobial samples were drawn from dialysis bag during 5-7 days to test for primary induction.

Fractionation: The plant supernatant was harvested (if acetylene reduction was detected in primary induction samples), filtered, centrifuged (at 20 000 xg per 20 mins), lyophilized and chromatographed on Sephadex G-25 (7). The individual fractions were filter-sterilized before being used in secondary induction experiments.

Nitrogenase assay: Nitrogenase activity was measured in rhizobial cultures sampled from dialysis bags or test rhizobia taken directly from suspension culture for primary or secondary activation, respectively, using acetylene dependent ethylene production as described previously (7). All the experiments were contaminant free.

Discussion: In primary induction experiments nitrogenase activity (up to 6 moles ethylene per day) during the initial 3-12 hours) was consistently obtained after 5-7 days co-culture.

In secondary activation experiments PCM promoted induction of nitrogenase activity in several strains of free-living rhizobia. Using unfractionated PCM, R.trifolii strain Tl Kan reduced as high as 543 moles ethylene viaI day. Fractionation of the crude PCM by Column chromatography (G25) showed that only certain fractions induced activity (these are called active fractions).

In conclusion, the use of white clover cell conditioned medium in liquid culture offers a reproducible technique for assessing nitrogenase activity in both slow and fast growing rhizobia and their mutants. The demonstration of white clover derived nitrogenase inducing factors (PCM) adds to the generality of in vitro induction of nitrogenase by legume cell cultures.

The economic value of legumes in terms of seed, forage and rotation crops has stimulated increased research into the cell culture systems of these species. Results obtained so far do not match the extent of cell culture technology attained with monocotyledons. However, progressing agricultural commitment to southern, new, chickpea and groundnut has caused many to increasing costs of nitrogenous fertilizer produced by energy-intensive industrial reduction have produced significant advances in terms of cell/suspension culture, plant redifferentiation, protoplast culture, somatic cell genetics and physiological application in herbicide research.

Despite these advances several recalcitrant species remain. The cell culture systems of perennial legumes that are more advanced than those of seed-legumes. This paper discusses the application of cell culture technologies to the economic improvement of legumes, while retaining some perspective toward required research commitments in agriculturally critical plant species such as soybean or groundnut. Subject areas discussed include: callus and suspension culture, plantlet differentiation and organogenesis, protoplast culture, cell fusion, cell culture application to study nitrogen fixation and nodulation, isolated root culture, and physiological studies using legume cell culture systems. Finally, immediate areas for legume cell culture application especially in mutation work are outlined.

INTRODUCTION

The values of tissue culture technology are three-fold:

The culture of sterile plant tissues as callus, suspension, protoplast or as isolated plant organ or developing shoot permits experimental analysis of external factors that influence the biological capability of the tissues. Current plant cell culture to a certain extent fulfils Haberlandt's (1902) postulate who stated that the culture of isolated tissues would permit the study of signals affecting differentiation and de-differentiation. The technology of isolated cell culture on defined media and their induction to differentiate into complete plants has resulted in a wide range of applications of cell culture in plant physiology, cell biology, genetics, cytology, biochemistry, microbiology, agronomy and horticulture.

The last 20 years of research produced a wide range of techniques which provide a solid basis for the application of cell culture in the propagation and genetic modification of crop plants. Figure 1 illustrates the relationship between conceptual fields of plant cell culture. The basic technologies have to be perfected before attempting research in the areas of propagation and genetic manipulation. Thus the boundary between basic and applied research often becomes diffuse as basic techniques can lead to immense economic and medical application. The technology of restriction nuclease biochemistry is an excellent example for this.

The fact that callus cultures or meristem/bud explants, under the right hormonal regime, can develop further into shoots and plantlets, coupled with the nearly clonal nature of many cell cultures, fuelled their application to clonal or vegetative propagation and their related technologies of virus elimination and meristem freeze preservation (Haskins and Kartha, 1980). This is especially of value for plant species which are recalcitrant to normal propagation practices. This symposium is replete with numerous examples of this type of application.

The last area of expansion falls under the heading of genetic modification and crop improvement, which is made possible by the microbial-like experimental system of isolated plant cells/protoplasts allowing the analysis of and selection from a large number of genetic units. Since each of these, as pointed out above, may retain the potential of plantlet formation, it is possible to mutate, select, hybridise, recombine and/or transform in cell culture, without losing the opportunity of testing the new culture of the whole plant stage.

Mutagenesis and selection in cell culture, however, presupposes the expression of the mutant allele. Conventional plant breeding technologies rely on malosis and segregation to observe mutant phenotypes in the F2 generation. Due to the absence of the meiotic reductional division in cell cultures, researchers have to (a) rely on dominant mutations (relatively rare), (b) use a system which allows the detection of mitotic recombinants of recessive mutations and their segregation products or (c) use haploid cell culture, derived from uninucleated pollen grains or pollen embryo/callus culture. Haploid cultures like most cell cultures are often plagued by ploidy instability.
APPLICATION OF LEGUME CELL CULTURE TECHNOLOGY

Legumes are an essential part of the world's agronomic system. Grazing agriculture heavily relies on forage legumes such as the clovers and alfalfa. Likewise seed legumes such as soybean, ground nut, kidney bean, mung bean, lentil, chickpea, cowpea, green gram and black gram are of great food and feed value, because of their high protein/oil content and the quality of protein, which due to its low sulphur-high lysine levels often complements the protein quality of cereals. Legumes fix atmospheric nitrogen through the root-nodule symbiosis involving a range of Rhizobium species (for a good reference text read Oudssel, 1974; Sprent, 1979 or Gibson and Newton, 1981). The global yield in terms of nitrogen fixed by legumes is estimated at 20 million tons per year.

Legume cell culture research clearly is sponsored by its agronomic importance. At the same time it is important to recognise that legume cell culture, although contributing to many facets of cell culture technology per se, has not yet provided results, which are directly applicable to agronomic systems. However, there are very few cell culture systems that can make this claim. Possibly tobacco, wheat, sugarcane, rice and possibly potato are initial successes in this direction (this list excludes, of course, those instances of ferns, ornamental or orchid propagation as well as the applications involving virus elimination).

Legume cell culture in contrast to other plant families except the cereals, had a slow start. Initial successes of callus and suspension cultures especially on Phaseolus and soybean failed to "close" the circle by developing differentiation systems. Early protoplast culture work lagged behind except for a few successes with soybean, Phaseolus and pea (see later discussion). Over the last few years reports on legume cell culture became more frequent and today several experimental systems (such as some clover and alfalfa) are at a stage, where agronomic application can be visualised.

Many of these advances, however, can also be attained by normal plant breeding methodologies. Cell culture
approaches in legumes (and this applies to other plant species as well) thus are not unique; it is hoped that the new technology can interact with the existing breeding practices. For example, it is possible to utilise media designed for somatic embryo culture to rear immature embryos of wide, interspecific hybrids produced by pollination.

Over the last twenty years legume cell and tissue cultures was significantly involved with the development of basic technologies. Emphasis focused on the development of culture systems could lead to the genetic improvement of the species. Little attention was given to that most legumes produce seeds which are not readily amenable to clonal propagation. Studies of differentiation and plantlet formation in legume cultures are focused on establishing the "connection" between the basic aspects and the genetic improvement of these crop species. Possibly the recognition of somaclonal variation as described by Krishnamurthi (paper on sugar cane in this volume), Shepherd et al (1980) and Scowcroft and Larkin (1981) for sugarcane and potato may increase the interest in vitro propagation as it may result in non-clonal variants being produced. These may aid breeding programmes, if shown to be stable, or may help in the genetic elucidation of phenomena such as symbiotic nitrogen fixation.

Which genetic improvement can one visualise for legumes? Increased yields, altered oil and protein content, morphological improvement, absence of non-palatable or toxic compounds, prolonged nodulation and nitrogen fixation capabilities, interspecific hybrids to overcome toxic compounds, prolonged nodulation and nitrogen fixation. Little attention was given to the fact that most legumes produce seeds which are not readily amenable to clonal propagation. Studies of differentiation and plantlet formation in legume cultures are focused on establishing the "connection" between the basic aspects and the genetic improvement of these crop species. Possibly the recognition of somaclonal variation as described by Krishnamurthi (paper on sugar cane in this volume), Shepherd et al (1980) and Scowcroft and Larkin (1981) for sugarcane and potato may increase the interest in vitro propagation as it may result in non-clonal variants being produced. These may aid breeding programmes, if shown to be stable, or may help in the genetic elucidation of phenomena such as symbiotic nitrogen fixation.

Which genetic improvement can one visualise for legumes? Increased yields, altered oil and protein content, morphological improvement, absence of non-palatable or toxic compounds, prolonged nodulation and nitrogen fixation capabilities, interspecific hybrids to overcome overcome predation, decreased blotting properties, and disease resistance. As such have been used as a basis for the detection of cytokinin activity, (see Miller, 1974). Blaydes (1966), for example, studied the influence of cytokinins and other hormone types on soybean cell growth. Bergman and Rannels (1975) pointed towards a source of artificial interpretation, when they showed that glutathione inhibited growth of soybean cell cultures used in cytokinin assay. Glutathione, they claimed, is a common component of plant extracts.

In the late 1960's and early 1970's the soybean cell line SB-1 was used extensively to determine nutritional requirements of plant cell cultures (Gamborg et al, 1968). Ohira and associates (Ohira et al, 1975; Ohjima and Ohira, 1977; Ohjima et al, 1975) used soybean suspension cultures to describe nutritional parameters controlling greening and iron uptake. Umetu et al (1975) showed that colchicine at low concentrations resulted in an increased separation of cells in soybean suspension cultures. Kang (1976) used this system to study potassium influences on nitrogen metabolism. Gresshoff (1979) cultured SB-1 soybean cells to elucidate the effect of the herbicide glyphosate (phosphono-methyl-glycine) on plant tissues and the reversibility of its inhibitory effect by phenylalanine and tyrosine. Similarly, Suen et al (1979) described amitrole effects on the nitrogen metabolism in soybean cultures. Chu and Lark (1976) looked at cell cycle parameters of soybean suspension cultures and emphasised its value as a system for genetic studies.

The ease of soybean suspension culture allowed the early development of protoplast isolation and culture techniques. Protoplast culture of soybean was reported (Kao et al, 1970). This was the first report of continued cell division obtained in protoplast derived cultures of any plant species. This protoplast experimental system of soybean was further applied to genetic studies in the Saskatoon laboratory. Ohyama et al (1977a, b) illustrated...
the uptake of exogenous DNA by nuclei isolated from protoplast preparations.

The protoplast system of soybean, however, provided greater promise for somatic hybridization research. Fowke et al. (1976) followed the ultrastructural events of soybean - sweet clover somatic hybrid formation and culture. Williamson et al. (1976) illustrated the same system that concanavalin A can be used to specifically mark the soybean plasma membrane. Fowke et al. (1977) continued their in depth ultrastructural research by following plastos in fusion hybrids between soybean suspension culture protoplasts and pea leaf mesophyll protoplasts. They indicated free mixing of cytoplasmic contents and chloroplast de-generation in hybrids. Williamson et al. (1977) showed ultrastructurally that wall regeneration as indicated by cellulose microfibril synthesis starts within 20–40 min. after enzyme removal. The fusion work reached a relative climax with the report by Kao (1977) of interspecific hybrid formation between soybean suspension protoplasts and Nicotiana glauca mesophyll protoplasts. Hybrid cell line were characterized by their variable retention of certain N. glauca chromosomes. The fusion hybrid failed to regenerate into whole plants. Wetter (1977) looked at isozyme patterns and correlated these with chromosomal loss in these soybean - N. glauca hybrids.

As yet there are no reports of mesophyll protoplast culture of soybean or of successful and repeated plant regeneration from soybean callus cultures. There are considerable difficulties in the isolation of protoplasts from soybean leaf tissue. Single cells are produced with relative ease (for example, a 2% pepticase treatment for 1–2 hours), which results in protoplasts beyond a yield of optimally 1% despite extensive attempts varying enzyme preparations, plant age, and plant conditions (watered vs. unwatered, light vs dark etc.). It should be stressed, however, that this situation is not restricted to soybean. Numerous tree species, for example, fail to yield protoplasts from leaf-tissue. Clearly such situations will require renewed investigative effort and some original thought. Observations by Zieg and Oatska (1980), however, provide some hope as these authors showed that soybean pod-tissue yields good protoplast preparations after a sequential incubation in 3% macerozyme for 2 h followed by 4 h in 5% cellulase R-10. The protoplasts were cultured to form callus tissues. Organogenesis other than occasional root formation was not observed.

Plantlet formation from soybean callus tissues is another example of the restrictive knowledge of plant tissue culture. The literature occasionally refers to soybean plantlet differentiation from hypocotyl- or cotyledon-derived callus (Kimball and Bingham, 1973; Beversdorf and Bingham, 1973; Oswald et al., 1977; Cheng et al., 1980). These reports, however, are always limited to cultures which still contain explant material and as such are not de-differentiated. In all cases it was noted that a few sub-cultures removed the organogenic potential. These facts coupled with the observations that established hypocotyl- and root-derived callus cannot form plantlets under a wide range of cell culture conditions and that newly induced hypocotyl callus occasionally produces a small plantlet, which always is associated with some inconspicuous callus-covered piece of explant material (compare to comments made by Beversdorf and Bingham (1977), Mott and Cure (1978)). Thomas and Wernicke (1978) and Kamiya and Widholm (1981) confirm the suggestion that differentiated soybean callus cultures cannot be regenerated into plantlets. Kamiya and Widholm (1981) support this claim in their study on plant regeneration from hypocotyl sections of several Glycine species. They furthermore extend their conclusion to the observed plantlet formation from 'callus' cultures of Pisum sativum derived from macerated shoot apices (Gamborg et al., 1974) and leaf (Malmberg, 1980).

The central thought of the criticism, however, can be applied to almost all regeneration systems, which (a) fail to go through the single cell stage or (b) tend to loose organogenic potential with continued subculture. Thus many claims of plantlet regeneration from legume cell cultures as those for Sisyrinchium hamatum (Scowcroft and Adamson, 1976), Lotus species (Nillzki and Grant, 1971), Phaseolus vulgarus (Gronomo et al., 1976a), peanut (Morgan et al., 1981) and many other claims of plant regeneration in other plant families, may fall into this category and extreme care needs to be taken to ensure the restricted interpretation of results. Regeneration attempts of established and cloned soybean cultures in our laboratory were restricted to callus hardening, extensive greening, vascularisation, woodiness, rhizogenesis, but never shoot, leaf or bud formation. A critical consideration in this regard is the "competence" of the explant tissue. Untested as yet are potentially morphogenetically competent tissues such as anther wall, apical buds, ovular and embryo tissues. Such tissues cultured on the appropriate media containing, for example, different nitrogen sources than nitrate may produce callus and suspension cultures of soybean (and possibly other legumes), which may reach their organogenic or embryogenic potential. The elegant study of Mullins and Shinvaan (1976) working with nucellar callus of grape vine (Vitis vinifera) should act as a guide to this type of work. Similarly work on scuttelar callus of Zea mays (Green and Phillips, 1975) and leaf base material of Sorghum (Wernicke and Brettell, 1980) indicated that the type of explant tissues can control the success of a cell culture as much as a phytohormone in a medium. Furthermore, ecotypic (ecotypic) differences as those pointed out by Malmberg (1980) in pea, Mok and Mok (1977) and Mok et al. (1980) in Phaseolus, Saunders and Bingham (1976), Bingham et al. (1975) and Keys and Bingham (1979) in Medicago arvina and Mokaputra and Greshoff (1981) in white clover illustrated the genetic control of tissue culture response.

Tissue specific responses were illustrated in our laboratory when working with white clover. Petiole explants of seedling material "behaved" differently in culture than root derived callus. Preliminary cytological studies point to some nucleotypic control of this phenomenon, as petiole callus was predominantly diploid (Zea 32) (see Fig. 3). While root callus showed a wide range of chromosomal variation ranging from a possible 22 to about 64 (note the sub-diploid ploidy of some cells). Polyploid material derived from root explants was expected as separate studies in collaboration with Dr. D. Castre and Dr. G. Peters (A.N.U.) on isolated protoplasts from distal tissues of white clover seedlings indicated that up to 60% of these cells contain nuclei which had twice the normal DNA content (Fig. 4a, b). These cells were not in a repli-
operative cycle as indicated by the complete lack of S-phase (intermediate DNA content) and absence of mitotic figures in over 1000 tested nuclei. Whether such cells are G2 arrested (i.e. after DNA synthesis but prior to mitosis), or are "true" G0 tetraploid cells is not known. However, this observation is of specific interest, as it (a) illustrated nucleotypic mosaicism within plant tissues and (b) gave a possible explanation to the fact that nodule tissue derived from cortical cell growth is of tetraploid nature (for further reading on this point, refer to Libbenga and Bogers (1974)). The former point may be of importance when considering the potential variability produced from cell cultures as described for white clover by Pelletier and Pelletier (1971) and more recently noted in sugarcane (see Krishnamurthi, this volume, and Scowcroft and Larkin, 1981) and potato (Shepherd et al, 1980).

The tissue culture of legumes, however, went beyond the development of the soybean and Phaseolus systems. For example, Grant and Fuller (1968) cultured root cells of Vicia faba, Graham (1968) established callus and suspension cultures of Trifolium subterraneum; Pelletier and Pelletier (1971) discovered large variability in terms of chromosome number and plant morphology. Bottino et al (1979) reported on the tissue culture of winged bean. Mroginski et al (1981) achieved the regeneration of peanut plantlets from callus cultures derived of immature leaves. Bingham (Saunders and Bingham, 1972; Saunders 1975; Bingham et al, 1975) and associates developed the Medicago system, which later led to detailed work into differentiation biology by Walker et al (1978, 1979) and protoplast culture and regeneration into complete plantlets (dos Santos et al, 1980; Kao and Michayluk, 1980). Shoot and bud differentiation was observed in cell cultures of Lathyrus sativus and Gec1 araritrum (Mukhopadhyay and Bhujwani, 1978, but see earlier note on differentiation). Plantlet regeneration was achieved in callus cultures of Stylosanthes hamata (Scowcroft and Adamson, 1976). Plant regeneration from callus is possible in red and crimson clover (Trifolium pratense and T. incarnatum) (Beshch and Smith, 1979; Phillips and Collins 1979a), meristem culture in red clover produced virus-symptom free plants of red clover (Phillips and Collins, 1979b), while somatic embryogenesis was seen by the same authors in the same species (Phillips and Collins, 1980). Meristem culture for propagation and cryopreservation studies has been reported by Kartha (see K. Karthe, this volume). Thus soybean, peanut and pea apical meristems can be cultured, stored and regrown at high efficiencies (see Haskins and Kartha, 1980; Kartha et al, 1981). Cousin et al (1979) pointed out that Vicia faba cell cultures are excellent material for the study of nuclear cytology in higher plants. Roeder (1979) described the cytology of callus and suspension cultures of Vicia faba. Ghosh and Sharma (1979) undertook cytological analysis of Vigna sinensis and Pimentum sativum cell suspension cultures.

Moharzadeh and Constantin (1978) presented a complete study of the tissue culture system (excepting protoplast culture) of Trifolium alexandrinum (berseem clover). They showed the possibility of callus and suspension cultures, plant regeneration as well as haploid culture via the anther culture-callus-plantlet route. Regrettably, there has been a failure to follow-up this type of work with further investigations and experimental applications. Extensive biochemical work was done on peanut callus cultures by Van Huyssee (1978) and Srivastava and van Huyssee (1977). Bajaj et al (1970) studying the sensitivity of Phaseolus vulgaris plants and tissues to X-rays found that callus cultures are significantly more radiation resistant. Mroginski et al (1981) and Sastri (personal communication) independently developed media and methods which allow

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**Fig. 4:** DNA content of clover protoplasts: A: Feulgen-microdensitometry of enzymatically produced, air-dried and Feulgen stained metaphyli pro­

**Fig. 5:** DNA content of clover protoplasts: A: Feulgen-
the regeneration of peanut calli into plants (but see earlier comment).

Haploid culture has had mixed successes in legumes. Mokhtarzadeh and Constantin (1978) were successful in *Trifolium alexandrium* resulting in plant regeneration. Niizeki and Grant (1971) working with *Lotus corniculatus* and *L. caudatus* var. japonicus. Gupta (1975) achieved haploid callus formation and plant regeneration in pea. Peters et al. (1977) reported haploid culture in *Phaseolus vulgaris*. Bajaj et al. (1980b) claim haploid embryo culture in *Calendula cajan* (pigeon pea) and pollen embryos and callus from anthers of *Arabidopsis thaliana*. However, the photographs presented substantiate only early cell divisions in pollen grains, but not embryogenesis.

**RECENT SUCCESSES TO DEVELOP LEGUME TISSUE CULTURE SYSTEMS**

Recent studies on forage legumes such as the clovers and alfalfa (*Medicago sativa*) have produced significant advances in the development of tissue culture systems so to allow the application of cell culture approaches in plant improvement.

Initial reports by Pelletier and Pelletier (1971) on white clover differentiation and cell culture variability, and Saunders and Bingham (1972, 1975) on alfalfa (indicating the potential to regenerate plantlets and the genotype control of this process) were extended to cover several perennial legume species. Phillips and Collins (1979a) reported the regeneration from callus culture of red clover (*Trifolium pratense*). This was followed by reports on virus-elimination after meristem culture (Phillips and Collins, 1979b) and a further report on somatic embryogenesis from cell suspensions (Phillips and Collins, 1980). Walker et al. (1978, 1979) studied the sequential nature of hormonal control in the organogenesis of alfalfa. Successful plant regeneration was obtained by Beach and Smith (1979) working with red and crimson clover. Thiamine (at 20 µmol/l) was found to aid organogenesis. As discussed above, Mokhtarzadeh and Constantin (1978) produced an extensive study on the cell culture of berseem clover. Gresshoff (1980) showed that protoplasts derived from suspension culture aggregates of white clover, could be cultured in hanging or sitting drops of culture medium to produce calli, which were capable of redifferentiation into complete plants (Fig. 2). Regenerated plants were shown to retain the diploid chromosome number of 32 (Fig. 3). Some variability especially in leaf shape (smooth versus rough margins), leaf number (one-, two- versus three- bladed) and petiole thickness were observed in cell culture regenerated tissues. Prolonged culture (either in suspension or callus) resulted in the loss of regeneration capability.

Similar successes were reported by Kao (1979), dos Santos et al. (1980) and Kao and Michaylyuk (1980) for the culture of alfalfa mesophyll protoplasts and their subsequent regeneration to plants. The work is of extreme significance as it couples the protoplast culture system with the observation of somatic embryogenesis.

The successful protoplast work in white clover and alfalfa follows a series of literature reports of protoplast culture from a wide range of legumes. In all these systems plant regeneration has not yet been achieved. Reports were restricted to protoplasts isolated from suspension cultures of soybean (Kao et al. 1974), *Phaseolus vulgaris*, *Pisum sativum* and *Vicia faba* (Gamborg et al., 1974) as well as shoot species of pea (Gamborg et al., 1975), and leaf tissues of *Pisum sativum* (Constand et al., 1973, Von Arnold and Eriksson, 1976), *Cicer arietinum* and *Melilotus alba* (Gamborg et al., 1974), *Phaseolus vulgaris* (Pelchers et al., 1974), *Vigna sinensis* (Davey et al., 1974), *Vicia faba* (Binding and Nehl, 1976a) *Vicia narbonensis* (Diron, 1976) and *Lathyrus aradorius* (Razdan et al., 1980). Pod tissue of soybean allowed the isolation and culture of protoplasts (Zieg and Outeka, 1960). Protoplasts were shown to form callus cultures. The report by White and Bhogwani (1981) on *Trifolium repens* is of particular interest as these authors managed to culture protoplasts derived from rapidly growing suspension cultures at plating densities of about 15 protoplasts per milliliter at efficiencies of 40–60%. It was essential to transfer after 4 days protoplasts with regenerated cell walls from a liquid medium to a solid medium with reduced (0.1 mg/l) instead of 2 mg/l 2,4-D auxin concentration. Regenerated protocellules were, unlike the parent suspension, auxin autotrophic, which may partially explain the inability by the authors to induce organogenesis.

**LEGUME CELL CULTURE: A STUDY OF SYMBIOTIC NITROGEN FIXATION**

(a) Nodule protoplasts. Protoplasts isolated from nodule tissues were shown to retain a marginal nitorgenase (acyetyl-lactone reduction) activity. Schetter and Hess (1977b) isolated root nodule protoplasts of soybean under anaerobic conditions showed optimally 2 nM ethylene per day per assay. Similar studies on nodule protoplast of *Vigna unguiculata* by Broughton et al. (1976), but using aerobic conditions, showed pronounced ATP effects after 96 hours of incubation (20 µmol ATP per 1.5 ml assay medium). optimum nitrogenase activity was about 12–13 µmol ethylene per mg dry weight per hour. Pretreatment with 10 µmol ATP and succinate (at 40 µmol/1.5 ml) stimulated observable activities. Both ATP and carboxylic acid effects point to the energy requirements of isolated nodule protoplasts. The authors furthermore suggested that nitrate in the incubation mixture needed to be metabolized before acetylene reduction could occur. A more complete study on *Vigna unguiculata* protoplasts was published by Wooi et al. (1981). These authors showed that although protein and RNA synthesis proceeding at a low rate, no DNA synthesis was observed. Gresshoff (unpublished data) detected nitrogenase activity in nodule protoplasts of *Trifolium pratense* after 24 h incubation.

Nodule protoplasts (Fig. 5a, b) of white and subterranean clover were used to ascertain whether nitrogen-fixing bacteria retain their forming capability if cultured on appropriate media (Gresshoff et al., 1977). These studies were extended to soybean (Gresshoff and Rolfe, 1978) and confirmed findings by Tsao et al. (1977). An advanced procedure for the isolation of protoplasts from white clover nodules was reported by Rolfe and Gresshoff (1980), who used this technique to facilitate the genetic interaction of mutant *Rhizobium* strains during the establishment of
nodulation. Isolated bacteroids of *Rhizobium trifolii* were characterized by their osmotic sensitivity, perhaps explaining earlier failures to obtain high viabilities of bacteroid suspensions (see Bergersen, 1968). Broughton (pers. comm.) has illustrated viability of *R. leguminosarum* bacteroids on osmotically buffered media but noted strain and host effect. Nodule protoplast isolation was reported previously by Davey et al. (1973). The fusion of such protoplasts with other protoplast types (e.g. suspension culture or mesophyll) was reported by Schetter and Hess (1977b), Syono et al. (1979) and Kajita (1980). The latter two publications focused on a fine structure analysis of fusion products of *R. leguminosarum* : is considered to be one of the most evolved symbiotic systems in nature. Many factors and the complexity of nodule at biochemical and ultrastructural levels. Therefore it seemed essential to develop an in vitro system to study the details of genetic fine structure and function of this association. Efforts have been made in the last decade in this direction, although the number of reports on cell culture associations are limited even now compared to those involving whole plants and micro-organisms. However, *in vitro* studies have made a noticeable contribution to our knowledge of the process of symbiosis. 

*In vitro* studies have been made with excised root cultures (Lewis and McCoy, 1933; Raggio et al. 1957) with detached rooted leaves, (Lie, 1971), with detached lupin root nodules (Sutton and Jepson, 1975) and with protoplasts (Schetter and Hess 1977b; Woni and Broughton, 1979 (see above)). Currently callus and cell suspensions along with rhizobia are being used as a model experimental system, particularly because they provide a more defined approach at the cellular-molecular level. Most of the discussions below are confined to this type of system.

Velicky and La Rue (1987) used cultured cells initiated from soybean root inoculated with *Rhizobium japonicum* ATCC 10254 and observed the stimulated lignification of cell walls and the differentiation of pitted cells in the tissue. It was suggested that these effects might be due to hormones produced by the bacteria, to a deficiency caused by microorganisms depleting the medium or to a non-specific reaction of plant tissues to microbial infection. However, in these experiments no evidence for intracellular bacterial symbiosis was obtained. Takatsu (1979) noted that co-culture of *Rhizobium japonicum* and soybean suspension cultures results in the release of cell division factors. These presumably are cytokinins which are synthesised by the bacterium.

Holsten et al. (1971) proposed the development of *in vitro* symbiosis to be analogous to that of intact root nodules. They had an experimental association comprising of soybean cell suspension culture inoculated with *R. japonicum* in multidiarm flasks rotated on a clinostat. Light and electron microscopy showed "infection-thread-like" structures containing bacteria, which penetrated the undifferentiated cell mass for considerable distances before releasing their contents into the cytoplasm of cultured cells. In some cases, the rhizobia were enclosed within a vesicle and, more interestingly, contained the inclusion polymer hydroxybutyrate, similar to that seen in bacteroids of the root nodule. This inclusion became more prominent with increasing time of inoculation of the synthetic symbiotic system (up to 15-20 days after inoculation). Although similar findings have been reported in *Arachis hypogaea*, *Cicer arietinum*, *Cajanus cajan* and *Pisum sativum* by Ranga Rao and Subba Rao (1976), the latter observed the bacteria entering the cells through the pores or gaps in the cell wall. The view that proper invasion occurred *in vitro* can be challenged in view of findings by E. Stenz (1971), who showed that cell cultures of pea, soybean and bean interact with *Rhizobium*, but that invasion was always restricted to a colonisation of dead cell spaces within the clumpy material. Reporter et al. (1975) using primary cultures of epidermal and cortical tissues of *Glycine max* var. Harosoy, observed a lag period of at least eight hours following inoculation before bacteria became attached to the plant cells. Mixing of bacteria only took place on certain of the soybean cells. The latter were observed by electron microscopy to secrete a filamentous pectinaceous material in response to the presence of *Rhizobium* which was responsible for binding the micro-organisms onto the walls.
However, many workers (such as Stent, 1971 and Verma et al., 1978) do not agree with Holsten's observation regarding invasion. It is not known whether the observed features of bacterial penetration and invasion of cells are a genetically controlled phenomenon or morphological artifacts created by simple mechanical penetration of bacteria. Verma et al. (1978) reported them as an abnormal infection (parasitic penetration) of the legume callus and suspension cells by comparing features of bacterial morphology and nitrogen fixation ability with normal infected tissue. Thus, the fact that normal symbiosis takes place in vitro, with sequential mechanisms similar to those observed in vivo, still remains obscure.

Soybean callus developed a brown colouration in response to infection by *Rhizobium* (Velicky and La Rue, 1967; Child and La Rue, 1974; Phillips, 1974a). This change is associated with cellular differentiation. Ranga Rao (1976) noted that organogenesis may also occur in cultures in the same medium remained undifferentiated. It was suggested that root formation occurred in response to kinetin and IAA produced by the bacteria. Later Hermina and Reporter (1977) claimed to stimulate differentiation of putative root hair cells by transfer of primary root cell cultures from medium containing 2,4-D and kinetin to medium lacking these hormones. The differentiated 'root hairs' could then be maintained in culture for long periods, when returned to medium with these growth regulators. The extent of response varied with the variety of soybean used (i.e. Acme tissues produced more root hair cells than those of Harosoy). Thus, such cultures with root hairs were considered more attractive for the study of bacterial attachment and infection than totally dedifferentiated cultures. The authors also observed that *Rhizobium* attached to the cells within three hours of inoculation in the medium containing low inorganic nitrate and lacking hormones. But the bacteria did not attach exclusively to root hair cells, confirming an observation previously made in vivo by Dart (1974). 'Root hair curling' or deformation of root was seen in less than 2% of the root hair cells.

An important observation common to all in vitro studies is that the pigment leghaemoglobin, normally responsible for the pink colouration of actively fixing root nodules has not been detected in cells cultured in association with *Rhizobium*. Bonartseva and Shemakhanova (1978) demonstrated nitrogenase activity in *Rhizobium* vigne (cowpea strain) and *Rhizobium meliloti* cultures maintained in a medium derived from Muraishige and Skoog (1962) containing 25mM arabinose, 25mM succinate and 2mM glutamine. This report, if substantiated, would be of interest as it shows that a fast-growing species (*R. meliloti*) of *Rhizobium* can be derepressed by the addition of plant culture metabolites.

Factors affecting nitrogenase activity in vitro association. Biological Factors. Holsten et al. (1971) showed that nitrogen-fixing potential of a symbiosis increased with the proportion of cellular invasion by microorganisms. Phillips (1974a) reported that cell aggregates of 0.5–2.0 mm in diameter were required for activity which might indicate that certain mass of tissue was necessary to protect nitrogenase from ambient oxygen. At the same time, Child and La Rue (1974) found that soybean cells induced nitrogenase activity in some strains of *R. japonicum*, whereas in contrast to Phillips' observation, the latter observed highest nitrogenase activity with *cowpea rhizobia*. Thus, host-symbiont genotypes seemed to play an important role in determining the level of detected activity. A broad correlation was found between the nitrogenase activity in nodules and in tissue culture association.

Most studies on *Rhizobium*-cultured cell associations have employed legume tissue of root origin. This is presumably related to the fact that root tissues are the site of symbiosis in the intact plant. Contrary to this hypothesis, Ranga Rao (1976) showed that callus initiated from stem tissue of *Stylosanthes gracilis* and inoculated with *Rhizobium* strain CB 552 showed more nitrogenase activity than tissue of root origin. He concluded that the observed activity might depend more on the physiological activity of the tissue at the time of inoculation than its genetic or developmental origin.

Environmental Factors. Holsten et al. (1971) noted that hormones such as 2,4-D and kinetin increased endogenous ethylene production of cultured cells, but decreased ethylene production as a result of nitrogenase activity which was later confirmed by Phillips (1974a). In contrast to these reports Child and La Rue (1974) found that 2,4-D or related chlorophenoxyacetic acids stimulated acetylene reducing activity.

A difficulty experienced by some workers appeared to be in relating their results to the results published earlier with respect to the content of inorganic nitrogen in the culture medium. Most found enzyme activity to be inhibited by inorganic nitrogen. In contrast, Child and La Rue (1974) who maintained inorganic nitrogen in the MS based medium at standard level, Child and La Rue (1974) reported a reduced level of N\(_2\)O and (NH\(_4\))\(_2\)SO\(_4\) of 1000 and 50 mg/l, respectively, in their BS medium. Phillips (1974a, b) omitted inorganic nitrogen completely and reported that NH\(_4\)Cl concentrations of 10mM or greater produced definite inhibition of acetylene reduction. Further investigation resulted in enhanced nitrogenase activity especially when nitrogen sources such as glutamine and citric acid cycle intermediates (such as succinate) were included in the agar medium (Child, 1975). In a subsequent study of infection of soybean variety Acme cells by *Rhizobium* strain 32H of the cowpea cross-inoculation group, Phillips (1974b) obtained a 16-fold stimulation of acetylene reduction by addition to the culture medium of glutamine and 4-enzyme bytrate (at 30mM nitrogen) or succinic acid as carbon source. Anderson and Phillips (1976) reported a stimulation of nitrogenase activity by proteins such as casein, BSA and hexane extracts of Glycine max, *Phasolus vulgaris* and *Plumb sativum*. Oxidized and autoclaved soybean meal also had a stimulating effect, the latter suggesting that protein breakdown products can be utilized by the *Rhizobium*-higher plant cell system.

The acetylene reduction activity normally lasted for 10–14 days period by G. max and R. japonicum, whereas in case of *Lupinus polyphyllus* callus infected with *R. lupini*, the acetylene reduction was detectable over an extended period of 4–6 weeks (Janner and Peters, 1975). In all cases, cultures were grown aerobically at 0.2 atm 0\(_2\).
which promoted nitrogenase activity. The conclusion was that the tissue culture cells were providing respiratory protection to the rhizobia. (a) Range of associations between cell cultures and rhizobia: *Rhizobium* associations with legumes like cowpeas (Sowcroft and Gibson, 1975), lupins (Werner and Ouberels, 1975), red clover (Ranga Rao, 1976) and soybean (Reeport and associates 1976–1978) have been reported. Child (1975) using strains of cowpea rhizobia and different sources of host cells (bean, sweet clover, *Vicia hajastana*) confirmed the earlier belief of Phillips (1974b) that strain 32HI can infect legume cells outside the cowpea group. More importantly, the ability of a strain to nodulate a host was not essential for the in vitro induction of nitrogenase in the bacteria. Of related interest was the observation of nitrogen-fixing association between auxotrophic *Azotobacter* and cultured carrot cells (nitrogen-starved) in which each symbiont was dependent on the other (Carlson and Chaleff, 1974, 1975).

To further develop the point of non-specificity, Child (1975) showed that *Rhizobium* strain 32HI reduced acetylene with tissue cultures of *Brassica napus*, *Bromus inermis* and *Trifolium monocaum*. At the same time, Sowcroft and Gibson (1975) illustrated nitrogenase activity of 32HI together with *N. tabacum* cv Wisconsin 38 which was later confirmed by 15N2 incorporation. The results obtained with tobacco were later confirmed by Gibson et al. (1976a). This was subsequently extended to association with tissue cultures of *carrot* (Ranga Rao, 1976), *Nemesis strumosa*, *Portulaca grandiflora* and *Petunia hybrida* (Schetter and Hess, 1977a). All workers except Ranga Rao (1976) showed that nitrogenase activity was observed with *N. tabacum* cv Wisconsin 38 which was later confirmed by 15N2 incorporation. The results obtained with tobacco were later confirmed by Gibson et al. (1976a). This was subsequently extended to association with tissue cultures of *carrot* (Ranga Rao, 1976), *Nemesis strumosa*, *Portulaca grandiflora* and *Petunia hybrida* (Schetter and Hess, 1977a). All workers except Ranga Rao (1976) showed that nitrogenase activity was observed with *N. tabacum* cv Wisconsin 38 which was later confirmed by 15N2 incorporation. The results obtained with tobacco were later confirmed by Gibson et al. (1976a). This was subsequently extended to association with tissue cultures of *carrot* (Ranga Rao, 1976), *Nemesis strumosa*, *Portulaca grandiflora* and *Petunia hybrida* (Schetter and Hess, 1977a). All workers except Ranga Rao (1976) showed that nitrogenase activity was observed with *N. tabacum* cv Wisconsin 38 which was later confirmed by 15N2 incorporation. The results obtained with tobacco were later confirmed by Gibson et al. (1976a). This was subsequently extended to association with tissue cultures of *carrot* (Ranga Rao, 1976), *Nemesis strumosa*, *Portulaca grandiflora* and *Petunia hybrida* (Schetter and Hess, 1977a). All workers except Ranga Rao (1976) showed that nitrogenase activity was observed with *N. tabacum* cv Wisconsin 38 which was later confirmed by 15N2 incorporation. The results obtained with tobacco were later confirmed by Gibson et al. (1976a). This was subsequently extended to association with tissue cultures of *carrot* (Ranga Rao, 1976), *Nemesis strumosa*, *Portulaca grandiflora* and *Petunia hybrida* (Schetter and Hess, 1977a).

The elucidation of exchange of substances in *Rhizobium*-legume symbiosis in vitro has undergone significant changes. However, to date, the PCM production and analysis of PCM components have only been done by Reporter and his associates. Many workers have realized that the technique used for PCM production, as described, does not work consistently. (see Gresshoff et al., 1981). There are many variables not yet clearly understood and it is not clear whether nitrogenase derepression by PCM components functions because of specific molecular signals or general physiological changes reminiscent of those inside a plant nodule.

**CONCLUSION**

The above review of legume tissue and cell culture and its application to study a range of biological phenomena was meant to serve as a guide through the literature. A certain degree of perspective (subjective as it may be) was introduced in the hope that future studies are not based on work which has not been viewed critically. Often information is distributed by review papers of this type; it is thus our opinion that the interested reader should refer to the original publication. With cell culture systems at a stage of development as outlined here, one can visualise the interaction of classical breeding methods with in vitro technology to facilitate the improvement of legume crops. Some comments passed in this review may be pessimistic — yet personal legumes over the last few years must stimulate further work into some of the more "different" legumes.

Note added in proof: Successful mesophyll protoplast isolation was achieved by Schwenk et al. (1981), who used mechanically isolated single cells from the primary leaf of soybean. A 99% yield was obtained after 60 min. digestion in pectolyase and cellulase at 30°C (Schwenk et al., 1981). Plant Sci. Lett. 23: 153–155.

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Ecotypic Variation of in vitro Plantlet Formation in White Clover (Trifolium repens)

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ABSTRACT

Sixteen Trifolium repens (white clover) genotypes of diverse geographic origin were tested for differences in organogenic potential of petiole and root derived explants. Significant differences were observed between ecotypes and explant types with respect to callus initiation and plantlet regenerability. The observed differences are attributed to existing genotypic variability as well as origin of explant tissue.

INTRODUCTION

Plant regeneration from tobacco and carrot callus cultures indicated originally that alterations of the hormonal environment were the major causative factors affecting this process. This concept has been confirmed now in over 1000 plant species. However, certain material remains recalcitrant to regeneration efforts despite extensive trial and error approaches. In several plant species varietal differences as well as differing explant tissues can affect the 'potency' of a plant culture in a particular medium. An example is the work by Mullins and Drinivasan (1976), who showed that macerated callus of Vitis vinifera (grape vine) is capable of somatic embryogenesis, although other tissues of the same plant failed to show any such response.

Similar tissue specific effects were seen with sorghum, where leafbase material retained morphogenic potential (Wernicke and Brettell, 1980) and maize, where acuticular tissue of a specific developmental stage is organogenic (Gengenbach et al, 1977).

Genotypic effects over cell culture responses have been noted in plantlet differentiation from callus cultures in peas (Halsberg, 1975), alfalfa (Saunders and Bingham, 1975; McCopp and Bingham, 1977 and Keys and Bingham, 1979), potato (Shepherd and Totten, 1977), red clover (Phillips and Collins, 1979) and from anther cultures in Arabidopsis (Gresshoff and Doy, 1971). The mechanism of this effect is not at all understood. However, Bingham et al. (1975) have shown that the tissue culturability of a cultivar is heritable. Similarly Mok et al. (1980) found that cell cultures of Phaseolus show a genotype specific difference in cytokinin metabolism which is reflected as cytokinin autonomy.

MATERIALS AND METHODS

(1) Callus induction

Seeds of 16 ecotypes of Trifolium repens (provided by Grassland Division, DSIR Palmerston North, New Zealand) were surface sterilized for 10 minutes at room temperature in 10% (vol/vol) saturated calcium hypochlorite (22ml) containing one drop of detergent (Sarkosyl N30, Ciba-Geigy) then rinsed in sterile water for 24 hours to allow inhibition in dark.

About 50 imbibed seeds from each ecotype were plated onto FM N03, (Fabaean medium containing 5mM KNO3, Rolfe et al; 1980) and were incubated at 25°C in light for germination. Root and petiole explants (0.3 – 0.8 cm long) were taken from healthy seedlings and tested for callus induction on CCM-1 (Table I). After two weeks resultant calli were excised from the original explant to CCM-2 (Table I) and were maintained by subculturing bi weekly. The term "passage" is used to indicate the number of subcultures after the initial callus induction step on CCM-1. All cultures were maintained in Nescofilm – sealed plastic petri-dishes (4-5 inocula/plate) at 25°C.

(2) Suspension culture

Rapidly growing, friable calli (1-2g) were macerated, then inoculated into 25ml of clover suspension medium-1 (Table I) and incubated at 25°C dark under gravity agitation (150 rpm). After 4-7 days of initial culture, a pipettable fraction was removed and subcultured into test media. Average growth rate in suspension was measured by using the method of settled cell volume determination (Gresshoff, 1979). This measure expresses the differences in settled cell volume detected during the culture period divided by the number of days. All the experiments involving growth kinetics were done under 25°C dark conditions.

To whom all correspondence should be addressed.
TABLE 1. Media Composition

<table>
<thead>
<tr>
<th>Code</th>
<th>Mineral Salts</th>
<th>Cytokinin</th>
<th>Auxin</th>
<th>% CH</th>
<th>% Agar</th>
<th>Culture conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCM-1</td>
<td>BS</td>
<td>0.5(Kin)</td>
<td>4(2,4D)</td>
<td>0.1</td>
<td>0.8</td>
<td>NCC(9L)</td>
</tr>
<tr>
<td>CCM-2</td>
<td>BS</td>
<td>0.5(Kin)</td>
<td>8(2,4D)</td>
<td>0.1</td>
<td>0.8</td>
<td>NCC(9L)</td>
</tr>
<tr>
<td>CCM-3</td>
<td>MS</td>
<td>0.15(Kin)</td>
<td>2(2,4D)</td>
<td>0.1</td>
<td>0.8</td>
<td>NCC(L)</td>
</tr>
<tr>
<td>CCM-4</td>
<td>MS</td>
<td>2(2,4D)</td>
<td>0.5(AA)</td>
<td>0.8</td>
<td></td>
<td>NCC(L-14)</td>
</tr>
<tr>
<td>CCM-5</td>
<td>MS</td>
<td>1(2,4D)</td>
<td>0.5(AA)</td>
<td>0.8</td>
<td></td>
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<tr>
<td>CCM-6</td>
<td>BS</td>
<td>0.1(1ip)</td>
<td>0.4(2,4D)</td>
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<td></td>
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<tr>
<td>CCM-7</td>
<td>MS</td>
<td>0.2(2,4D)</td>
<td>0.2(2,4D)</td>
<td>0.8</td>
<td></td>
<td>NCC(L-14)</td>
</tr>
</tbody>
</table>

* CCM = clover callus medium; CDH = clover differentiation medium; CSM = clover suspension medium; NCC = normal culture conditions; media contain vitamins, iron chelates, trace elements, ZU sucrose, pH 5.8 - 6.0 all as described by Grishoff (1980); DL = diffuse light; L = continuous light; L-14 = 14 h light and 10 h dark; Kin = kinetin; 2-ip = 2-isopentylaminopurine; 2,4-D = 2,4-dichlorophenoxy acetic acid; MS = major salt formulation of Murashige and Skoog (1962); BS = major salt formulation of Hurashige and Skoog (1962), CSH = major salt formulation of Gamborg and Eveleigh (1968), CH = casein hydrolysate.

using 1:4 (V/V) dilutions of the pipettable fraction. Growth data are averages of experiments followed over two successive subculturings.

(III) Organogenesis

Essentially the transfer schedule of Grishoff (1980) was followed to induce plantlet formation and regeneration of whole plants using clover differentiation medium CDM-1 through to CDM-7.

RESULTS AND DISCUSSION

Callus was initiated on both ends of petiole explants on CDM-1 and rapidly proliferated up to 1g fresh weight within two weeks. On subculturing onto CCM-2, growth centres (compact nodules of meristem-like cells) were observed in calli of ten genotypes (Table 2), eventually giving rise to green nodules, roots (with root hairs) and green shoots with varying number of leaves (1 to 3). The calli from six other genotypes (Table 2), which failed to differentiate under the conditions tested, maintained the usual callus growth rate. Direct differentiation into shoots on callus growth medium during the second callus subculture passage was of interest. The plantlets of five ecotypes formed on second passage callus adapted quickly (within three weeks), by forming complete root systems allowing explanting into soil on being transferred to CDM-3. Thus, the plant regeneration period was considerably reduced for these genotypes as compared to the method previously described by Grishoff (1980). Two genotypes (C2634, C2734) retained the capacity for differentiation on the high auxin medium up to the sixth passage.

In contrast to calli from petiole explants, the calli from root explants of different ecotypes resulted from general proliferation of the entire explant tissue. These tended to show differing growth rates. On average, root calli grew better than calli from petiole explants. Root calli from all different ecotypes were similar in morphology. Spontaneous rhizogenesis was observed only in three ecotypes (C2994, C2568 and C256J). Calli from those ecotypes along with the non-differentiating calli from petiole explants were sequentially transferred to a differentiation media (starting with CDM-1 through to CDM-7) to encourage plantlet differentiation. The regeneration into complete plants was only possible in three genotypes (C2634, C2734 and C2126). Other calli lines showed early stages of differentiation, but may require different hormonal or nutritional conditions to facilitate complete differentiation. Some ecotypes failed to show any obvious differentiation response.

In general, petiole calli showed increased morphogenic response compared to root calli, although a variable ability to differentiate was observed among ecotypes. Petiole wound callus of white clover, in general, has higher 'competence' than root callus isolated under identical exogenous hormonal conditions. Whether lowered hormonal levels would allow the isolation of callus from root tissue that still maintains 'competence' was not tested.

Calli of both root and petiole origin from eight ecotypes (Table 2) were subcultured into clover suspension medium (CMM-2) to study differential growth capability. Suspension cultures of any particular ecotype were initiated after differing times of callus (solid medium) culture.

Generally the growth rate was slower in cell suspension from petiole calli compared to root calli. The observed differences in growth rates and morphogenic potential were maintained through repeated callus induction. Variability among individual seed isolates from any one cultivar was less pronounced than those observed between cultivars. Owing to the heterozygosity of white clover, however, variability within any cultivar
TABLE 2. Differentiation, plantlet formation and callus and suspension growth of white clover ecotypes

<table>
<thead>
<tr>
<th>Ecotypes Place of Origin (grassland number)</th>
<th>Petiole explants</th>
<th>Root explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>differentiation capability</td>
<td>growth rate</td>
</tr>
<tr>
<td>C2094 France</td>
<td>G +++</td>
<td>0</td>
</tr>
<tr>
<td>C2122 Denmark</td>
<td>R +++</td>
<td>0</td>
</tr>
<tr>
<td>C2126 England</td>
<td>F +</td>
<td>PD</td>
</tr>
<tr>
<td>C2157 Belgium</td>
<td>R +++</td>
<td>0</td>
</tr>
<tr>
<td>C2564 Portugal</td>
<td>P +R</td>
<td>PD +</td>
</tr>
<tr>
<td>C2568 Netherlands</td>
<td>R +</td>
<td>R</td>
</tr>
<tr>
<td>C2569 Brazil</td>
<td>G +++</td>
<td>0</td>
</tr>
<tr>
<td>C2584 Lebanon</td>
<td>P+RS +</td>
<td>PD +</td>
</tr>
<tr>
<td>C2634 Spain</td>
<td>RS +</td>
<td>0 +</td>
</tr>
<tr>
<td>C2636 USA</td>
<td>R +</td>
<td>R</td>
</tr>
<tr>
<td>C2734 Kenya</td>
<td>P+R</td>
<td>RS +</td>
</tr>
<tr>
<td>C2994 India</td>
<td>R +</td>
<td>0 +</td>
</tr>
<tr>
<td>C3551 Costa Rica</td>
<td>R +</td>
<td>0 +</td>
</tr>
<tr>
<td>C3557 Latvia</td>
<td>R +</td>
<td>O</td>
</tr>
<tr>
<td>C6487 New Zealand</td>
<td>G +++</td>
<td>0</td>
</tr>
<tr>
<td>C6583 New Zealand</td>
<td>G +++</td>
<td>0</td>
</tr>
</tbody>
</table>

NT = not tested; G = green nodules capable of regeneration; R = rhizogenesis only in first passage; RS = rhizogenesis in more than one sub-culture; P = plantlet formation and direct plant regeneration on induction medium; PD = plant regeneration on differentiation media; 0 = lack of differentiation as described; += slow callus growth (usually less than 1g per week from 25-30 mg (fresh weight) inoculum); +++= rapid callus growth (usually more than 1g per week from 25-30 mg (fresh weight) inoculum).

In some ecotypes (such as C2634, C2734 and C2569), cells in suspension exhibited rhizogenesis. Ecotype C2634 and C2734 retained rhizogenicity for four subcultures. Roots formed during the last subculture were devoid of root hairs. Abnormal 'embryos' (these failed to develop further) and disorganised cell clumps ultimately giving rise to roots were observed in petiole cell suspension of C3551, C2634, C2734 and C2569. Such events occurred only in slow growing cultures. The rootlets from these suspensions, on being transferred to root culture media grew into normal roots.

The above data illustrate that cell lines isolated from ecotypes C2634 and C2734 maintain a high morphogenic potential under a wide range of culture conditions. Further studies on mesophyll protoplast isolation and plant development should focus on such organogenic donor material.

The present study emphasises the importance of genetic as well as tissue specific control of callus growth and differentiation of white clover in vitro. Attention needs to be paid not only to the medium used during organogenic induction, but, as was shown here, the nature of the explant (i.e. tissue, organ, growth stage, or ecotype) and the nature of the callus induction medium (resulting in preferential cell proliferation) are important to allow the successful initiation of organogenic pathways.

REFERENCES

Malmberg RL (1979) Planta 146 : 243-244