dons, however is not affected by chloramphenicol[16] while in rice[17,18] and maize seedlings[19] it is induced by the antibiotic. Dixit *et al.*[20] reported the inhibition of induction of nitrate reductase by chloramphenicol in maize leaves. Such conflicting results exist in the literature. The present study therefore suggests the need for investigating and understanding the effect of chloramphenicol directly or indirectly on eukaryotic protein synthesis inhibition.

TABLE 3

Effect of chloramphenicol on cytosolic isocitrate dehydrogenase malate dehydrogenase and isocitrate lyase activities from Aspergillus niger

Conditions of the growth	Isocitrate dehydrogenase*	Malate dehydrogenase*	Isocitrate lyase*
Control	13.9	556.2	34.8
With chloramphenicol (2 mg/ml)	6.6	328.4	18.4
With chloramphenicol (4 mg/ml)	5.6	206.7	18.5

*(units/mg protein)

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1. Gale, E. F. and Folkes, J., *Biochem. J.*, 1953, **53**, 493.
2. Mager, J., *Biochem. Biophys. Acta.*, 1960, **38**, 150.
3. Robson. R. and Novelli. G. C., *Proc. Natl. Acad. Sci.*, 1960, **46**. 484.
4. Stoner, C. S., Hodge, T. K. and Hanson, J. B., *Nature*, (*London*) 1964, **203**, 258.
5. Anderson, L. A. and Smillie, R. A., *Biochem. Biophys. Res. Commun.*, 1966, **23**, 535.
6. Shah, V. K. and Ramakrishna, C. V., *Enzymol.*, 1963, **25**, 367.
7. Jagannathan, V., Singh, K. and Damodaran, M., *Biochem. J.*, 1956, **63**, 94.
8. Ochoa, S., *Meth. Enzym.*, 1955, **1**, 735.
9. Dixon, G. H. and Kornber, H. L., *Biochem. J.*, 1959, **72**, 3.
10. Bernfeld, P., *Meth. Enzym.*, 1955, **1**, 49.
11. Warburg, O. and Christian, W., *Biochem. Z.*, 1938, **296**, 150.
12. Srivastava, B.I.S. and Meredith, W.O.S:, *Can.J. Bot.*, 1962, **40**, 1257.
13. Young, J. L. and Varner, J. E., *Arch. Biochem. Biophys.*, 1959, **84**, 71.
14. Slutters, Scholten, C.M.Th., *Planta.*, 1973, **113**, 229.
15. Veevers, L., Schrader, L. E., Flesher, D., and Hageman, R. H., *Plant Physiol.*, 1965, **40**, 691.
16. Knypl, J. S., *Z. Pflanzer Physiologie.*, 1973, **70**, 1.
17. Shen, T. C., *Planta.*, 1972, **21**, 108.
18. Shen, T. C., *Physiol.*, 1972, **49**, 546.
19. Sohrader, L. E., Beevers, L. and Hageman, R. H. Biochem. *Biophys. Res. Commun.*, 1967, **26**, 14.
20. Tripathi, R. D., Srivastava, H. S. and Dixit, S. N., *Indian J. Exp. Biol.*, 1977, **15**, 939.

REGULATION OF NITROGENASE EXPRESSION IN *RHIZOBIUM*

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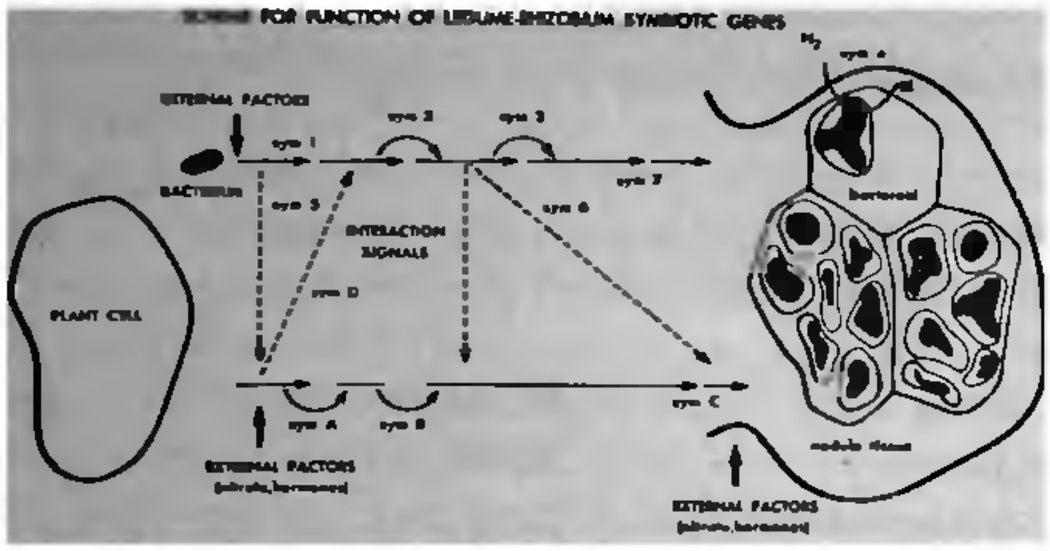
INTRODUCTION

THE catalytic reduction of atmospheric dinitrogen to ammonia by nitrogenase and its subsidiary enzyme systems (nitrogen fixation), is of immense agricultural importance. Research on biological nitrogen fixation was pioneered by intensive studies of legume-*Rhizobium* symbioses, since legumes contribute more to the nitrogen economy of the world than any other system. Reductionist efforts to characterise these symbioses have been made with excised root cultures[1,2], detached leaves and pods[3], nodules[4], nodule protoplasts[5]; and isolated bacteroids[6]. Although potentially valuable, the application of these isolated experimental systems is limited because of the enormous complexities involved in the symbiosis. Development of an effective symbiosis comprises a multi-step cascade of events, namely root colonisation, root hair adhesion, infection, nodule initiation and development, bacteroid development and ultimately nitrogen fixation[7]. This requires a tightly co-ordinated reciprocal communication between plant and bacterium[7,8] as shown in figure 1. The range of non-fixing *Rhizobium* mutants isolated[9]



TWO DEVELOPMENTAL PATHWAYS OF DIFFERENTIATION WITH TIGHTLY COORDINATED RECIPROCAL INTERACTION

Figure 1: A scheme for function of Legume-*Rhizobium* symbiotic genes. The success of symbiotic process requires the co-ordinated read-off of genetic signals between plant and the bacterium, both being subjected to control by environment. Plants cells undergo differentiation (Sym. A, B etc) for cortical via meristematic to bacteroid filled cells. Meanwhile the vegetative bacterial cells undergo a morphogenesis (Sym. 1, 2 etc) and develop into nitrogen fixing bacteroids.

indicates that each step of the symbiosis is governed by at least one prokaryotic gene. Further complications at different biochemical and physiological levels arise in the study of the nodule, an organ *sui generis* often plagued by changes in macro and microenvironmental conditions[10]. Thus, isolated plant organs have a restricted value for the study of the central features of the symbiosis such as the expression of nitrogenase.

The last decade has seen considerable progress towards an understanding of the more subtle aspects of the nitrogenase regulation. Attempts have been made to mimick the plant-*Rhizobium* symbiosis in *ex planta* co-cultures of plant cells and *Rhizobium*. Additionally, under appropriate conditions nitrogenase activity (the final operative phenotype in the root nodule symbiosis previously thought to be expressed only in bacteroids) has been demonstrated in axenic cultures of *Rhizobium*. Although it is not known yet, whether or not such asymbiotic activity by *Rhizobium* occurs in the rhizosphere, this discovery was significant as it permitted the development of the genetics of *Rhizobium* nitrogenase and its regulation. This article discusses these developments.

IN VITRO SYMBIOSIS

Recent advances in cell and tissue culture opened up new avenues for studying symbiotic nitrogen fixation at the cellular level. Co-cultures of plant callus or cell suspension with rhizobia were initially used as model systems. Progress in this area has been recently reviewed[11,12] and is summarised in table 1.

(a) *Infection and nodulation:*

Original interest in *in vitro* co-culture was directed towards its potential as a system to study the infection process. Although many workers demonstrated *Rhizobium* infection of plant cell cultures to be similar to the *in planta* situation (table 1), others[20-22] have referred to the phenomena as a superficial, morphological artifact rather than a genetically-controlled developmental process. Furthermore, the behaviour of cultured plant cells does not always correspond to the genetic constitution of the plant from which they were derived. Thus, the conclusions drawn from infection studies made *in vitro* were anomalous. Ability of *Rhizobium* strains to interact with legume stems[32] and non-legume derived callus culture[33] conclusively demonstrated that genetic barriers to *in planta* nitrogen fixation (infection, nodule initiation, etc.) either were not operable or were bypassed *in vitro*. The recent discovery of promiscuous *Rhizobium* strains (belonging to the cowpea miscellany) capable of nodulating legume stems[33] and various non-leguminous tree species (belonging to the *Parasponia* genus)[77] shows a relaxed stringency of plant—*Rhizobium* association similar to *in vitro* studies. These findings have thus helped to dispel the existing dogma that a *Rhizobium* strain is capable of nitrogen fixation only when associated with the root of particular legumes defined by its cross inoculation specificity.

(b) *Nitrogen fixation in vitro*

The capability of *Rhizobium* strain 32H1 to derepress nitrogenase activity (as measured by acetylene reduction or $^{15}N_2$ incorporation) in the presence of both legume and non-legumes callus and later in the absence of any plant cells elicited two important features: (a) the genetic information for nitrogenase is encoded in the bacterium and (b) that the diffusable but yet nutritionally substitutable substances produced by the plant cells were probably responsible for stimulating *Rhizobium* nitrogenase activity. Interestingly some of the nutritional and environmental factors controlling success of the *in planta* symbiosis also regulate expression of nitrogenase *in vitro*. For example, nitrate, ammonia and glutamine as well as oxygen have repressing effects. Succinate, a carbon metabolite passing from the plant cytoplasm to the bacteroid , was a key compound to supporting high levels of *in vitro* nitrogenase activity. Further investigations using a trans-filter apparatus (*i.e. Rhizobium*-plant cell suspension co-cultures)[28] extended these initial observations made on separated agar cultures.

Nitrogenase activity was derepressed and/or stimulated in normally non-derepressable or moderately

TABLE 1

Summary of nitrogen fixation studies in cell culture-Rhizobium associations

Associations involved	Aspects of symbiosis studied	References
1 *Rhizobium*-legume associations		
(1) *R. japonicum-Glycine max*	(a) Cellular differentiation	13, 14, 15, 16, 17
	(b) Intracellular symbiosis	18, 19, 20
	(c) Nutritional and cultural parameters:	
	Hormonal	13, 14, 15, 16, 17, 18, 22
	inorganic combined N_2	15, 16. 17, 18, 23, 24, 25, 26
	Carbohydrates	17, 27
	(d) Host symbiont genetic effect	15
	(e) Stimulatory/inhibitory effect of diffusable factors	17, 25, 28, 29, 44, 45, 46
	(f) Non-specificity	29. 30
(2) *R. vigna–Arachis hypogaea* (cowpea miscellany)	Intracellular symbiosis	31, 32
— *Stylosanthes gracilis*	non-specificity (stem callus used)	33
— *Glycine max*	non-specificity	27, 34
— *Pisum sativum*	(differential cross inoculation group	
— *Trifolium* Spp	combinations)	
— *Vicia hajastana*		
— Cowpea	Stimulatory effects of diffusable factors	34
(3) *R. trifolii - Trifolium*	Organogenesis and cellular differentiation	33
- T. repens	Stimulatory effects of diffusable factors	36
(4) *R. lupini - Lupinous polyphyllus*	Stimulatory factors	37
2. *Rhizobium* (strain 32H1) - Non-legume associations		
— *Brassica napus*	deviation of	
Bromus inermis	*Rhizobium* - legume	34
Tritieum monococcum	symbiosis *in vitro* and stimulatory effects of diffusable factors	
— *Nicotiana tabacum*		35, 39, 40
— *Nemesia strummusa*		35, 39, 40
Portulaca grandiflora		38, 41
Petunia hybrida		33
Daucus carota		48
Triticum aestivum		48
Sorghum nigricans		

derepressed *Rhizobium* strains during co-culture with plant cells. This effect was also obtained when vegetative *Rhizobium* cells were exposed to co-culture supernatant. It was postulated that this phenomenon involved an exchange of molecular signals between plant and bacterial cells. Recently legume cell cultures of soybean, pea, and white clover were shown to respond to certain bacterial substances by synthesizing dialysable plant factors (also called nitrogenase factors) which were claimed to accumulate in the conditioned plant cell medium (PCM)[29, 30], subsequently causing derepression of nitrogenase.

Fractionation of plant cell conditioned medium indicated that only certain fractions were able to stimulate expression of nitrogenase activity in pure cultures of *Rhizobium*. PCM fractionation led to the

postulate by Reporter that *cupper*-containing metallothioneins and peptidoglucans were involved in the *in vitro* interaction[42]. PCM was reported to increase oxidative phosphorylation[43]. In parallel, the derepression of nitrogenase was apparently associated with the loss of exopolysaccharide (Reporter, personal communication). However, there are many gaps, inconsistencies and experimental weaknesses that cloud the reliability and interpretation of the above mentioned studies and the numerous related investigations by other workers. It remains obscure in the absence of reproducible results, whether PCM components represent complex molecular signals. Whether such 'signals' exist, or if they are a collection of 'single' organic molecules involved in normal cellular metabolism still remains unclear, although the *in vitro* culture on completely defined media of some strains (*e.g.* CB756, ANU289 or 32H1) indicated that at least for some strains there was no need for signal complexity.

Nitrogenase In Free-Living Rhizobium

Although attempts to show asymbiotic nitrogen fixation by rhizobia were first documented in 1945[49], the conclusive evidence for such activity was not obtained until 30 years later. The reports on *in vitro* symbiosis between plant cells and rhizobia (table 1) provided the primary impetus to efforts and subsquently discovery of nitrogen fixation in free-living rhizobia[50-52]. The demonstration of nitrogen fixation by $^{15}N_2$ incorporation[51,52] and nitrogenase activity (acetylene reduction) inhibition by specific inhibitors[53] has confirmed the early observations. Since then either agar, stationary/shaken liquid or chemostat culture of *Rhizobium* in defined media has been used to produce nitrogenase activity. To date more than 50 strains (table 2) show the nitrogenase positive phenotype under *in vitro* conditions.

However, most of these strains belong to slow growing *Rhizobium* species such as *R. japonicum* and 'cowpea strains'. Several strains belonging to these species cannot be derepressed for nitrogenase under similar conditions as used for derepressable strains. Since the strains differ with regard to the requirements for expression of nitrogenase activity[54-56], it may be that yet optimal conditions necessary for derepression of nitrogenase in these strains have not been found. Alternately, derepression of nitrogenase in the laboratory cultures may be under genetic control. The results of some relatively recent genetic exchange experiments[57] and DNA:DNA hybridization studies[58] indicated that strains labelled '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

Table 2

Number of strains tested for the ability to reduce acetylene in free living state in various Rhizobium species.

Rhizobium species	Nitrogenase positive		Nitrogenase negative	
	exclusively agar	liquid as well as agar	agar	liquid
'Cowpea' strains	12	4	9	—
R. japonicum	8	26	5	13
R. lupini	—	—	4	—
R. meliloti	—	2(?)	4	2
R. trifolii	—	2(?)	—	16
R. leguminosarum	1(?)	—	6	—
Parasponia Rhizobium	1	1	14(?)	—

—, not known
(?), not confirmed results

nitrogenase positive in vitro[65] it was thought that nitrogenase derepression in culture may be confined to one sub-species. The organisation of nitrogen fixation genes was reported to be very different in *R. japonicum* strains USDA110 and 61A76[75], belonging to the same sub-species (on the basis of DNA homology classification), of which only strain 61A76 is capable of nitrogenase expression *in vitro*. Thus it appears that the genetic basis for *in vitro* nitrogenase expression may be strain specific. The precise molecular architecture for such differences between strains remains to be worked out.

In contrast, as shown in table 2 there are only a few unsubstantiated reports in the literature of nitrogenase activity with fast growing *Rhizobium* species. Demonstration of nitrogenase activity in *R. trifolii* strain T1, the spectinomycin resistant derivative T1 spec[61] or by exposure to plant cell conditioned medium[36] was not repeatable in our laboratory and in that of others (Beringer pers. comm.). Furthermore, other reports of nitrogenase activity in fast growing *Rhizobium* strains[59,60] are either characterised by a lack of repeatability or proper contamination tests. It is thus our opinion that as yet a repeatable derepression of nitrogenase in fast growing *Rhizobium* strains such as *R. melilotii*, *R. trifolii* and *R. leguminosarum* under defined or in associated *in vitro* culture is not demonstratable.

Several factors influence derepression of nitrogenase in *Rhizobium in vitro*. Some recent reviews[55, 62, 63] have discussed these factors. Here, we discuss some new developments which have not been reviewed previously.

(a) *Regulation by Oxygen*

Nitrogenase activity on agar cultures was measured under atmospheric oxygen tension (0.2 atm), which was later found to be the optimal concentration[52]. Attempts[52] to derepress nitrogenase in liquid culture under air were unsuccessful as a consistently low O_2 tension was obligatory for derepression. Oxygen concentrations in the range of 0.06 to 0.36% were effective[56, 64]. Studies in chemostat cultures of strain 32H1 indicated that cultures growing with about 1 micromole dissolved oxygen gave high specific rates. The optimal level of oxygen in the gasphase differed with carbon and nitrogen sources used in the medium[65, 66] Different strains showed variable O_2 optima, all being within a small range generally classified as microaerobic[56].

It was not known then, whether the lack of activity under aerobic conditions was due to O_2 repression of nitrogenase synthesis, O_2 inactivation of nitrogenase or auto-oxidation of reduced electron donors. Measurement of antigenically cross-reacting material[66] in aerated continuous cultures of strain 32H1 showed oxygen repression of nitrogenase synthesis. Exposure to moderate oxygen concentrations (20–30 μM dissolved oxygen tension) of the culture with established nitrogenase activity also resulted in inactivation of nitrogenase[66]. Similar effects of O_2 on nitrogenase synthesis were observed by pulse labelling derepressed cells with a ^{14}C labelled amino acid in free-living *R. japonicum*[67]. In *Klebsiella pneumoniae* oxygen is thought to interact with the *nif* LA regulatory protein complex which in turn controls transcription of the *nif* HDK operon responsible for the synthesis of the Fe and Mo/Fe component protein of nitrogenase. Whether O_2 regulates nitrogenase synthesis in *Rhizobium* in a manner similar to that observed in *Klebsiella* remains to be answered.

(b) *Regulation by Carbon sources*

Rhizobium strains differ in their preference for a carbon source for growth as well as derepression of nitrogenase. Initially a combination of two carbon sources (a pentose sugar, *e.g.* arabinose and a TCA cycle intermediate, *e.g.* succinate) was advocated[39, 40, 54] to derepress nitrogenase activity in strain 32H1. From our work with *Parasponia-Rhizobium* strain ANU289, it appears that of the two, succinate seems to play a dominating role in derepression. Comparable results were also obtained[56] using a single carbon source (gluconate or succinate) in strain 32H1 and several *R. japonicum* strains. However, it must be noted that the later reports utilised the liquid derepression rather than agar culture system as well as different oxygen levels.

The exact role of a carbon source in derepression of nitrogenase is yet not understood. Using an experimental approach that allows the quantitative determination of the *de-novo* biosynthesis of the constituent polypeptides of nitrogenase, the amount of synthesis was shown to be dependent upon the carbon source used in free living *R. japonicum*[67]. 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 catalytic activity of nitrogenase depends on the supply of reducing equivalents and energy, the carbon source may exert its effect by modulating general metabolism of the cell[55]. A negative correlation between exopolysaccharide production and nitrogenase activity was found in liquid cultures in a survey of 20 *Rhizobium* strains[66]. It was envisaged that nitrogenase and exopolysaccharide (EPS) synthesis compete for energy (in energy limiting microaerobic conditions). Thus in strains, which produce large amounts of EPS, most available energy was utilized in the production of EPS rather than synthesis and maintenance of nitrogenase. EPS synthesis and nitrogenase depression in *Parasponia-Rhizobium* strain ANU289 (non-mucoid on mannitol containing medium) and its isogenic mucoid derivative strain ANU288[56, 77] supported the above hypothesis[65]. Further studies with inhibitors specific for exopolysaccharides synthesis or further isolation of specific mutants such as strain ANU288 and ANU289 lacking any one of the enzymes necessary for exopolysaccharide synthesis may be helpful in understanding the correlation between EPS synthesis and nitrogenase activity.

(c) *Regulation by ammonium*

Rhizobium strains with the exception of *Sesbania* (Dreyfus, pers. comm.) strains in contrast to other free-living nitrogen fixing organisms exhibit the nitrogen fixing phenotype only in a developmental state which is different from their normal vegetative growth state. Attempts to grow *Rhizobium* on its own fixed nitrogen have been difficult because derepression of nitrogenase occurred only in the presence of a utilizable nitrogen source, such as glutamate, glutamine, ammonium chloride, potassium nitrate, aspartate,

asparagine and casaminoacids. The type and amount of the particular nitrogen sources varies between strains.

For example, inhibition of nitrogenase activity by 10 mM ammonium was observed in agar culture[40, 60] at 20% oxygen in the gas phase. In contrast, similar concentration of ammonium had no inhibitory effect on nitrogenase in strains 32H1 and 31-1b-83 in dilute shaken culture under low oxygen tension[64]. Thus a close interaction between oxygen and ammonium concentrations and culture method was thought to be involved. Furthermore, studies with *R. japonicum* strain 31-1b-83 revealed that the degree of ammonium inhibition was pH dependent and the maximum inhibition was found at the optimum pH for nitrogenase activity[55]. Variable effects of ammonium depending upon the carbon source used in the medium observed in our laboratory indicated that the inhibition may be mediated by a possible metabolic effect. Thus the oxygen and carbon effects can be explained by differential growth and thus depletion. As ammonium effects on nitrogenase activity are strain specific and affected by carbon sources, oxygen levels, culture regime and the presence of other nitrogenous compounds, it is at present difficult to develop a generalised mechanism of ammonium regulation in *Rhizobium*. In the absence of evidence for lack of polypeptide synthesis, it is not known whether inhibition is due to repression of nitrogenase synthesis or simply a modulation of acti vity. More recently, some new findings on ammonia assimilation in *Rhizobium* have been reported. Glutamine (Gln) auxotrophs of *Rhizobium* strain 32H1 failed to derepress synthesis of nitrogenase both in culture and *in planta*[69]. The nitrogen fixation (*nif*) defects in these strains were shown to be the direct result of glutamine auxotrophy as reversion to prototrophy simultaneously recovered nitrogenase derepression ability.

The current model which states that unadenylated GS mediates derepression of nitrogenase[70] is consistent if not an extrapolation by hyperbole, with the regulation of GS by the classical adenylylation cascade established by Stadtman and his colleagues for *E. coli*[71], and more recently in *Klebsiella*[76]. Regulation of other *nif* operons in *K. pneumoniae* by the *nif* LA operon which is subjected to control by products of *gln* operons is shown in figure 2. Whether the regulatory patterns as shown for *Klebsiella* also hold true for *Rhizobium* in general is still unknown. Perhaps arguing against a general concept is the fact that (a) *Rhizobium* has two GS (GSI + GSII) enzymes, (b) the sequence homology of the regulatory region of *K. pneumoniae nif* HDK is minimal, compared to that of *R. meliloti* and *R. trifolii* and (c) in some *Rhizobium*

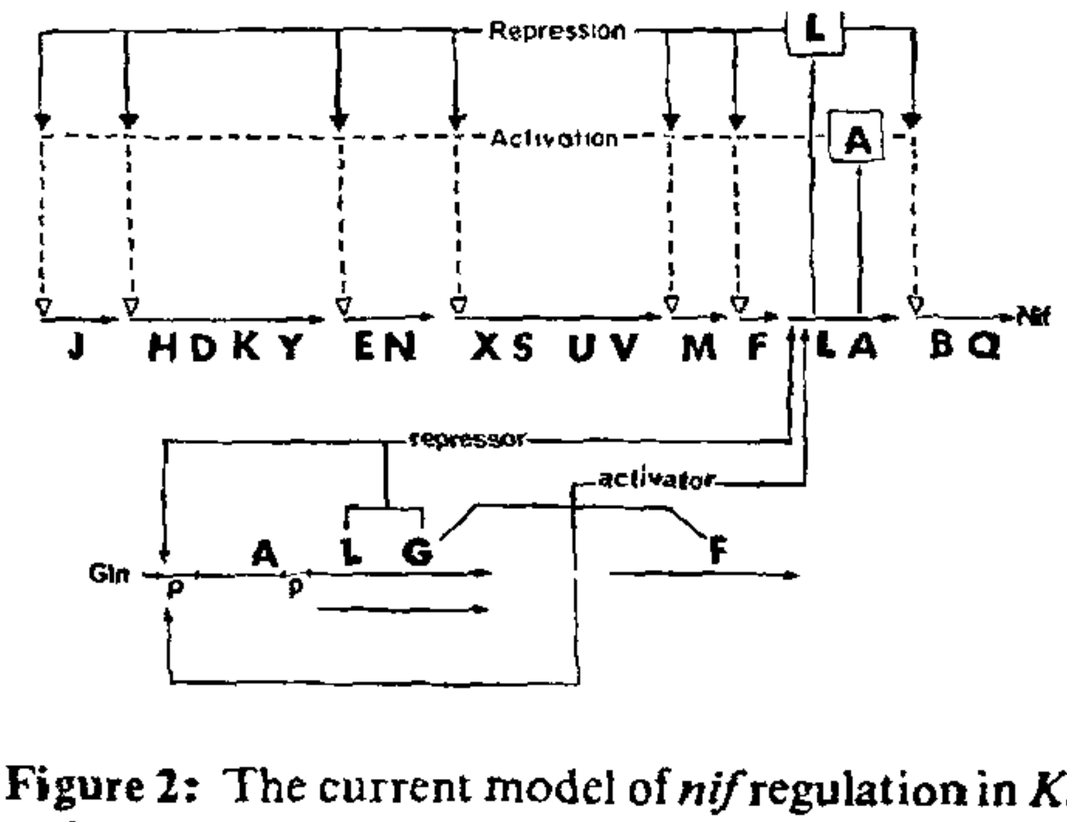


Figure 2: The current model of *nif* regulation in *Klebsiella pneumoniae*. General nitrogen control, genes such as *gln G* (ntr C), *gln F* (ntr A) and *gln L* (ntr B) are involved in regulation of *nif*. *gln F* product activates *gln G*, which in turn activates transcription from *nif LA* promoter. *gln L* product either by itself or in combination of *gln G* repress *nif LA* transcription. In *nif* cluster, *nif L* and *nif A* gene act respectively to repress (solid line) or activate (dotted line) all other *nif* operons.

strains (notably *Parasponia-Rhizobium* strain ANU289) the *nif*H (Fe-protein) and *nif*D (component of the Mo–Fe protein) are not in the same transcriptional unit as they are either in *K. pneumoniae* or *R. trifolii* and *R. meliloti*. (Scott and Shine, Personael Communication).

Before one builds models for *Rhizobium* nitrogenase regulation based on *Klebsiella* data, it is essential that more fundamental *Rhizobium* work is carried out.

Concluding Remarks

In essence, the so-called reductionist approach using *in vitro* co-cultures of plant callus-cell suspension with rhizobia has not provided such a simlified experimental system compared to the intact nodule as was formerly anticipated. The approaches outlined however permitted an elaboration of factors controlling nitrogenase activity *in vitro* and ultimately led to the discovery that the genes for nitrogenase are encoded by the *Rhizobium* genome. Additionally, the derepression of nitrogenase activity in free-living *Rhizobium* has undoubtedly initiated investigations aimed towards the understanding of several aspects of regulation of nitrogenase and related assimilatory enzymes.

Understanding the expression the *Rhizobium* nitrogenase, has recently acquired a commercial character stimulated by the need to increase protein

production and to reduce the use of expensive inorganic fertilizers on a world-wide basis. The advent of new recombinant DNA techniques has advanced our knowledge of *Rhizobium* participation in symbiosis. For example some of the genes responsible for both nodulation and nitrogen fixation processes have been identified and cloned[72-74]. The physical mapping of *nif* genes in *Rhizobium* is currently undertaken in several laboratories. Whether our increased understanding of the molecular biology, genetics and biochemistry of nitrogenase and symbiotic nitrogen fixation actually will result in increased crop production or just constitute a further indepth analysis of a complex developmental process is beyond the scope of this paper.

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1. Lewis, K. H. and McCoy, E., *Bot. Gaz.*, 1933, **95**, 316.
2. Raggio, M., Raggio, G. and Torrey, J. G., *Am. J. 1957*, **44**, 325.
3. Lie, T. A., *Plant Soil*, 1971, **34**, 663.
4. Sutton, W. D. and Jepsen, N. M., *Plant Physiol.*, 1975, **56**, 665.
5. Wooi, K. C., Bisseling, T., Kammen, A. V. and Broughton, W. J., In *Advances in protoplast research*, (eds) L. Ferenczy and K. L. Farras, 1980, p. 441.
6. Bergersen, F. J., In *The biology of nitrogen fixation*, (ed.) A. Quispel, 1974, p. 473.
7. Vincent, J. M., In *Nitrogen fixation* II, (eds) W. E. Newton and W. H. Orme-Johnson, *Univ. Park Press*, Baltimore, 1980, p. 103.
8. Nutman, P. S., In *Nitrogen fixation*, eds. W. D. P. Stewart and J. R. Gallan, Cambridge Univ. Press, London, 1980.
9. Rolfe, B. G., Djodjevic, M., Scott, K. F., Hughes, J. E., Jones, J. B., Gresshoff, P. M., Cen, Y., Dudman, W. F., Zurkowski, W. and Shine, J., In *Current perspectives in N_2-fixation*. (eds) A. H. Gibson and W. E. Newton, *Aust. Acad. Sci.*, Canberra, 1981.
10. Carroll, B. J., Honours thesis submitted, pp. 142 to Botany Department, Australian National University, 1980.
11. Davey, M. R. and Cocking, E. E., In *Recent advances in biological nitrogen fixation*, (ed.) N. S. Subba Rao, Oxford and IHB Publishing Co., New Delhi, 1980, p. 281.
12. Gresshoff, P. M. and Mohapatra, S. S., In *Proc. Symp. on Plant Tissue Culture of Economically important Plants*, (ed.) A. N. Rao, Singapore, p. 11.
13. Velicky, I. and La Rue, T. A., *Naturwissenschaften*, 1967, **54**, 96.
14. Takats, S. R., *Z. Pflanzenphysiol.*, 1979, **95**, 105.
15. Child, J. J. and La Rue, T., *Plant Physiol.*, 1974, **53**, 88.
16. Phillips, D. A., *Plant Physiol.*, 1974, **54**, 67.
17. Hermina, N. and Reporter, M., *Plant Physiol.*, 1977, **59**, 97.
18. Holsten, R. D., Burns, R. C., Hardy, R. W. F. and Herbert, R. R., *Nature* (*London*), 1971, **232**, 173.
19. Holsten, R. D. and Hardy, R. W. F., In *Methods in enzymology: Photosynthesis and nitrogen fixation*, Part B, (ed.) A. San Pietro, Acad. Press, Zentralblatt, 1972, **44**, 497.
20. Stenz, E., *Zentrablatt for Bakt Parasitenkunde Infektionskrankheiten and Hygiene*, Zweite-naturwiss Abteil, 1981, **126**, 142.
21. Verma, D. P. S., Huner, N. and Bal, A. K., *Planta*, 1978, **138**, 107.
22. Child, J. J. and La Rue, T., In *Proc. 1st Int. Symp. on Nitrogen Fixation*, eds. W. D. Newton and C. J. Nyman, Pullman, Washington 1976, Washington State University Press, pp. 447.
23. Phillips, D. A., In *Proc. 1st Int. Symp. on Nitrogen Fixation*, eds. W. E. Newton and C. J. Nyman, Pullman, Washington, 1976, Washington State University Press.
24. Anderson, S. J. and Phillips, D. A., *Plant Physiol.*, 1976, **57**, 890.
25. Werner, D., Wilcockson, J. and Kalkowski, B., *Z. Naturforsch*, 1975, **30**, 687.
26. Werner, D., Ber. Deutsch, *Bot. Ges. Bd.*, 1976, **89**, 563.
27. Phillips, D. A., *Plant Physiol.*, 1974b, **54**, 654.
28. Reporter, M., *Plant Physiol.*, 1976, **57**, 651.
29. Bednarski, M. A. and Reporter, M., *Appl. Environ. Microbiol.*, 1978, **36**, 115.
30. Gresshoff, P. M., Carroll, B., Mohapatra, S. S., Reporter, M., Shine, J., and Rolfe, B. G., In *Current perspectives in nitrogen fixation*, eds. Gibson, A. H. and Newton, W. E., *Aust. Acad. Sci.* Canberra. 1981, pp 209.
31. Ranga Rao, V. J., Sopory, S. K., and Subha Rao, N. S., *Curr. Sci.*, 1974, **43**, 503.
32. Ranga Rao, V. J. and Subha Rao, N. S., *Z. Pflanzenphysiol.*, 1976, **80**, 14.
33. Ranga Rao, V. J., *Plant Sci. Lett.*, 1976, **6**, 77.
34. Child J. J. *Nature* (*London*), 1975, **253**, 350.
35. Scowcroft, W. R., and Gibson, A. H., *Nature* (*London*), 1975, **253**, 51.
36. Mohapatra, S. S., Rolfe, B. G., Reporter, M., and Gresshoff, P. M., In *Proc 1st Aust. Nat.*

Symp. on Plant Cell ana Tissue Cultue, eds. J. Hutchinson, and P. M., Gresshoff, 1981, p. 19.
37. Werner, D., and Oberlies, G., *Naturwissen* schaften, 1975, **62,** 350.
38. Lusting, B., Plischke, W. and Hess, D., *Z. Pflanzenphysiol.*, 1980, **98,** 277. 7.
39. Gibson, A. H., Child, J. J., Pagan, J. D., and Scowcroft, W. R., *Planta*, 1976a, **128,** 233.
40. Gibson, A. H., Scowcroft, W. R., Child, J. J., and Pagan, J. D. *Arch. Microbiol.*, 1976b, **108,** 45.
41. Schetter, C., and Hess, D., *Plant Sci. Lett..*, 1977, **9,** 1.
42. Storey, R., Rainer, K., Pope, L. and Reporter, M., *Plant Sci. Lett.*, 1979, **14,** 253.
43. Reporter, M. Skotnicki, M. L., and Rolfe, B. G. *Aust. J. Biol. Sci.*,1980, **33,** 613.
44. Ozawa, T., and Yamaguchi, M., *Agrol. Biol. Chem (Tokyo)* 1981, **45,** 413.
45. DeMoranville, C. J., Kaminski, A. R., Barnett, N. M., Bottino, P. J., and Blevins, D. G. *Physiol. Plant*, 1981, **52,** 53.
46. Gresshoff, P. M., In *Proc. Int. Sym. Plant Cell and Tissue Culture* Kyoto, 1982.
47. Lusting, B., Plischke, W., and Hess, D., *Z. Pflanzenphysiol.*, 1980, **98,** 277.
48. Hess, D. and Kaiefer, S., *Z. Pflanzenphysiol.*, ,1980, **101,** 15.
49. Virtanen, A. I. Jorma, J., Linokola, H. and Linnasalmi, A., *Acta Chem. Scand.*, 1947, **1,** 90.
50. La Rue, T. A., Kurz, W. C. Z. and Child, J. J., *Can. J. Microbiol.*, 1975, **21,** 1884.
51. McComb, J. A., Elliot, J. and Dilworth, M. J., *Nature*, 1975, **256,** 409.
52. Pagan, J. D., Child, J. J., Scowcroft, W. R. and Gibson, A. H., *Nature* (*London*), 1975, **256,** 406.
53. Keister, D. L., *J. Bact.*, 1975, **123,** 1265.
54. Pankhurst, C. E., *J. Appl. Bacteriol.*, 1981, **50,** 45.
55. Keister, D. L. and Ranga Rao, V., In *Recent development in nitrogen fixation*, (eds).W. E., Newton, J. R. Postgate, and Rodrigeuz-Barrueco, Acad. Press, 1977, pp, 419.
56. Mohapatra, S. S. and Gresshoff, P. M., In *Proc 12th Int. Cong. Biochem.*, Perth, 1982, p. 398.
57. Johnston, A. W. B. and Beringer, J. E., *Nature* (*London*), 1977, **267,** 611.
58. Hollis, A. B., Kloos, W. E. and Elkan, G. H., *J. Gen. Microbiol.*, 1981, **123,** 215.
59. Bedmare, E. J. and Olivers, J., *Curr. Microbiol.*, 1978, **2,** 11.
60. Lorkiewicz, Z., Russa, R. and Urbanik, T., *Acta-Microbiol. Polonica.*, 1978, **27,** 5.
61. Skotnicki, M. L., Rolfe, B. G. and Reporter, M., *Biochem, Biophys. Res. Commun.*, 1979, **86,** 468.
62. Child, J. J., In *Recent advances in biological nitrogen fixation*, Ed. Subha Rao, N., New Delhi, 1980, pp. 325.
63. Eady, R., In *Current perspetives in nitrogen fixation*, eds. A. H. Gibson and W. E. Newton, *Aust. Acad. Sci.*, 1981, p. 172.
64. Keister, D. L. and Evans, W. R., *J. Bact.*, 1976, **129,** 149.
65. Agarwal, A. K. and Keister, D. L., In *Proc 8th North American Rhizobium Conference*, 1981.
66. Bergersen, F. J., Turner, G. L., Gibson, A. H., Dudman, W. F., *Biochem. Biophys. Acta.*, 1976, **444,** 164.
67. Scott, D. B., Hennecke, H., Lim, S. T., In *Proc. 7th North American Rhizobium Conf.*, 1979.
68. Kurz, W. G. W. and La Rue, T. A., *Nature* (*London*), 1975, **256,** 407.
69. Ludwig, R. A. and Signer, E. R., *Nature* (*London*), 1977, **267,** 245.
70. Ludwig, R. A., In *Microbiology, Am. Soc. Microbiology*, (ed.) D. Schlessinger, Washington, 1981, p. 93.
71. Stadtman, E. R. and Ginsberg, A., In *Enzymes*, Vol. 10, 3rd Ed., (ed. P. D. Boyer), Acad. Press, New York, 1974, pp. 755.
72. Beringer, J. E., *J. Gen. Microbiol.*, 1980, **116,** 1.
73. K. F. Scott, Hughes, J., Gresshoff, P. M., Beringer, J. E., Rolfe, B. G., Shine, J., *J. Mol. Appl. Gen.*, 1982, **1,** 315.
74. Ausubel, F. M., *Cell*, 1982, **29,** 1.
75. Haugland, R. and Verma, D. P. S., *J. Mol. Appl. Genet.*, 1981, **1,** 205.
76. Ow, D. W. and Ausubel, F. M., *Nature* (*London*), 1983, **301,** 307.
77. Mohapatra, S. S., Bender, G. L., Shine, J., Rolfe, B. G. and Gresshoff, P. M., *Arch. Microbiol.*, 1983, (In press).