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# The Molecular and Genetic Manipulation of Nitrogen Fixation

J. R. Postgate and F. C. Cannon

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# The molecular and genetic manipulation of nitrogen fixation

By J. R. Postgate, F.R.S., and F. C. Cannon
*A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton, BN1 9RQ, U.K.*

The fundamental importance of dinitrogen fixation for world agriculture, in relation to projected energy supplies, population pressure and food requirements over the next decades, obliges scientists to reconsider ways of exploiting this biological process. Genetic manipulation offers several options in principle. Existing symbiotic systems such as the legumes and seemingly inefficient systems such as the grass associations could be improved; new symbioses could be developed by *nif* gene transfer to rhizosphere commensals or by somatic hybridization of appropriate plants. A major advance would be to render plants independent of microbes by manipulation of expressable *nif* into the plant genome. This goal is discussed. It requires the complete genetic and physical characterization of *nif*, in particular its regulation, and an understanding of the physiological background within which *nif* can be expressed, as well as the ability to fuse *nif* to alien genetic systems. Substantial progress in these directions has been made by using the *nif* genes of *Klebsiella pneumoniae*; this progress is reviewed. Strategies for the further manipulation of *nif* towards regulated expression in the plant genome are considered.

## Nitrogen fixation

The transcendent importance of nitrogen fixation in sustaining the biosphere has been recognized for much of the present century. In the last two or three decades the world's human population has outstripped the ability of natural nitrogen fixation processes, spontaneous and biological, to support adequate food production, so that more than 30% of the world's population now depends on artificial N fertilizer for its minimal nutrition. Documentation of the quantitative and scientific bases of these assertions is widely available (see, for example, Postgate (1980*a*, *b*) and references therein). Essentially, nitrogen fixation returns to the biosphere nitrogen atoms that have been lost into the atmosphere as a result of denitrification. Both nitrogen fixation and denitrification are primarily biological processes, though chemical processes supplement them on a global scale, and both are exclusive to prokaryotic microbes: the bacteria. (One instance of nitrogen fixation by a eukaryotic microbe has been reported by Yamada & Sakaguchi (1980), but the physiology and taxonomic status of this interesting green alga is not clear.)

Energy and transport costs of the Haber–Bosch process, as well as threats of environmental catastrophe from greatly augmented use of chemical N fertilizer make further exploitation of chemical N fertilizer an unattractive solution to world food problems. This conclusion has excited much interest in the biological process, and many proposals for more extensive exploitation of biological nitrogen fixation have been presented. Though N fertilizer use is inescapable, and greater exploitation of existing diazotrophic systems will certainly come about, even together they will not solve the problem of feeding the human population of the world by the early decades of the next millennium; more imaginative developments, involving genetic and somatic manipulation of existing and newly developed systems, will be necessary (see Postgate (1980*b*) for references to this discussion).

## Options

For this contribution we shall restrict our presentation to the improvement of existing systems involving plants, or the development of new plant systems, and shall say nothing of the possible exploitation of microbes *per se*, of the nitrogenase enzyme or of chemical catalysts based thereon.

### *Improved systems*

Legumes, which form diazotrophic associations with bacteria of the genus *Rhizobium*, are the most important of the agricultural crops that are capable of exploiting biological nitrogen fixation. Plants such as peas, pulses, beans, lucerne and clover can make substantial contributions to food and fibre production with minimum N fertilizer costs as long as the plant and environment are appropriate. Other diazotrophic associations are exploited to a lesser extent. The efficacy of the *Rhizobium*–legume association, and in principle of all diazotrophic associations, depends on a variety of factors, some of which are amenable to genetic manipulation. A few illustrative examples follow.

#### *Competitiveness*

'Wild' rhizobia are often relatively ineffective symbionts in the sense that they transfer little fixed N to the plant, yet they often out-compete more effective strains which have been introduced exogenously. Genetic manipulation (e.g. transfer of effectiveness genes to highly competitive strains) would improve this situation.

#### *Hydrogenase*

Possession of an uptake hydrogenase by the rhizobia augments the efficiency of the soybean symbiosis by a mechanism that is partly understood (see Evans *et al.* 1980). Mutants in the genes for hydrogenase (*Hup*$^-$ mutants) are available and the manipulation of these genes into strains of rhizobium with other virtues will become possible. Some species of *Rhizobium* (e.g. *R. leguminosarum*; Ruiz-Argueso *et al.* 1978) seem always to be deficient in hydrogenase, a situation that ought to be genetically correctable.

#### *Fixation period*

In many legumes, nitrogen fixation ceases before pod-filling, and manipulation of the plant or bacteria to prolong the fixation period would enhance crop yields (Hardy 1976).

#### *The plant host*

Breeding of plants for selectivity towards effective rhizobia and/or for enhanced uptake of $CO_2$ (and transfer of photosynthate to the symbiont) are examples of prospects for genetic manipulation of the plant partner of the legume symbiosis (Hardy 1976).

#### *Cereal associations*

Diazotrophic associations of *Azotobacter* (Dobereiner *et al.* 1972), *Azospirillum* (Dobereiner & Day 1976), *Bacillus* (Larson & Neal 1978) and other diazotrophs (Barber *et al.* 1978) with the roots of grasses or cereals are well established. Such diazotrophic rhizocoenoses (Dobereiner &




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De-Polli 1980) are relatively ineffective, but genetic manipulation of both plant and microbe ought to yield agronomically more important associations.

Extensive discussion of the possibilities for generating improved systems, which should cover actinomycete and cyanobacterial associations (see Sprent 1979) as well as those mentioned above, is inappropriate to this paper.

### *New systems*

The generation of new diazotrophic systems has been an attractive long-term proposition since the demonstration of intergeneric *nif* gene transfer among enteric bacteria (Dixon & Postgate 1972), and several new species of diazotrophic bacteria have been generated by conjugational transfer of laboratory-constructed *nif* plasmids (see Krishnapillai & Postgate 1980). Generation of new symbioses is a far more complex process and may be approached in three major ways.

1. Transfer of *nif*, and any other ancillary genes required for regulated expression, into organisms that already have close mutualistic associations with plants. In fact, such associations often exist where the selection pressure is favourable, as in the grass rhizocoenoses mentioned above. Transfer of expressible *nif* to mycorrhiza is an interesting possibility of this kind (see Giles & Whitehead 1975), but requires sustained and regulated expression of essentially prokaryotic genes in a eukaryotic background; this problem ought not to be insurmountable.

2. Somatic hybridization (see Cocking, this symposium) of appropriate plants to generate new hybrids that combine agricultural desirability with diazotrophic capability. Such projects do not involve genetic manipulation of *nif* and we shall not discuss them further.

3. Introduction of expressible *nif* into the plant genome. This approach, if successful, would have the important advantage that the plant would not only become diazotrophic in its own right, like a highly effective diazotrophic symbiosis, but it would also become independent of a prokaryotic symbiont, and thus unconcerned by problems of effectiveness and competitiveness which complicate the husbandry of existing diazotrophic associations.

This third approach can be subdivided. One plan would be to seek a benign prokaryotic endosymbiont capable of diazotrophy which would establish itself as a new diazotrophic organelle-like structure, analogous to a chloroplast. We are unable to prescribe a systematic strategy for the construction of a stable, regulated diazotrophic organelle so we shall not discuss this matter further. The transfer of *nif* genes from a diazotroph, together with ancillary genes, into some pre-existing part of the plant genome can be approached systematically. It is a long-term project, since it absolutely requires a solution to the problem of expression of a complex of prokaryotic genes in a eukaryote, but it is also the most challenging, since present knowledge of biological nitrogen fixation indicates no reason why it should not be successful. Before discussing strategies for achieving this option, we shall briefly indicate the present state of knowledge of the *nif* genes, their expression and manipulation.

## Progress

### Nif *genes in* Klebsiella pneumoniae

The close linkage of *his* and *nif* on the *K. pneumoniae* chromosome facilitated the construction of self-transmissible plasmids that carry the *his–nif* region (Cannon *et al.* 1976; Dixon *et al.* 1976). The plasmid pRD1 has the genetic properties of its precursor, the P-incompatibility group plasmid RP4, and also carries the chromosomal genes *gnd rfb his nif shi*A. This plasmid and

several derivatives have been used for complementation analyses and fine structure mapping of *nif* mutations. pRD1 has also been used to investigate the expression of *Klebsiella nif* genes in other bacterial genera (Dixon *et al.* 1976; Cannon & Postgate 1976; Postgate & Krishnapillai 1977; Krishnapillai & Postgate 1980) and was the source of DNA for cloning the *nif* genes.

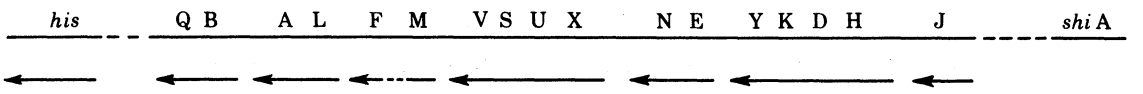


FIGURE 1. Map of *Klebsiella pneumoniae nif* genes (MacNeil *et al.* 1978; Merrick *et al.* 1980; Riedel *et al.* 1979; Puhler & Klipp 1981). The arrows indicate *nif* operons and their directions of transcription.

Seventeen contiguous *nif* genes have been identified and mapped close to the *his* operon (figure 1). Fourteen of these were recognized by complementation analysis, and further genetic characterization of mutations from one of the complementation groups suggested that it was composed of two genes, *nif*A and L (MacNeil *et al.* 1978; Merrick *et al.* 1980). This was confirmed by the identification of the polypeptides specified by *nif*A and *nif*L in strains carrying *nif*AL gene clones (Puhler & Klipp 1981; F. C. Cannon, unpublished). The gene order, *his*... *nif*QBALFMVSUNEKDHJ was unambiguously established by deletion mapping (MacNeil *et al.* 1978; Merrick *et al.* 1980). Two additional genes, *nif*Y, located between *nif*E and K, and *nif*X, located between *nif*U and N (see figure 1), were identified on the basis of polypeptide elimination in *Escherichia coli* minicells carrying *nif* gene clones with Tn5 insertions. The locations of *nif*X and Y were determined by physical mapping of the Tn5 insertions (Puhler & Klipp 1981). Complementation analysis of insertion mutations that exert transcriptional polarity indicated that the *nif* genes were arranged in seven or eight operons and that the multicistronic operons were transcribed in the same direction as the *his* operon (figure 1). RNA–DNA hybridization studies also showed that *his*-specific and *nif*-specific mRNA synthesized *in vivo* at detectable levels under the conditions used were transcribed from the same DNA strand (Janssen *et al.* 1980).

## Nif *gene clones*

The location of *nif* genes in a single cluster linked to *his* on the plasmid pRD1 facilitated their molecular cloning by the construction of a series of small plasmids carrying overlapping DNA restriction fragments that collectively cover the entire *nif* region (Cannon *et al.* 1977, 1979). Puhler *et al.* (1979*a b*), using a different strategy, have also constructed clones that carry all the genes of the *nif* cluster, and MacNeil & Brill (1980) have isolated *in vivo* a series of λ*nif* transducing phages, which collectively carry the complete cluster of *nif* genes.

The sites at which several restriction endonocleases cleave the *nif* region were mapped by using the *nif* gene clones and this allowed the assignment of *nif* insertion mutations (induced by translocatable genetic elements) to physical locations with respect to the restriction sites (Cannon *et al.* 1979; Riedel *et al.* 1979). The physical *nif* map derived from these studies is in complete agreement with the genetic map. A more detailed physical map of the *nif* genes and an unambiguous identification of most of their products has been obtained by mapping the sites of Tn5 insertions derived by 'saturation mutagenesis' of *nif* gene clones and monitoring any resulting changes in number and/or size of *nif*-specific polypeptides synthesized in *E. coli* minicells (Puhler & Klipp 1981). The size of the *nif* region is approximately $23 \times 10^3$ base pairs and the results of physical mapping suggest that there are no gaps in the current *nif* map.




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TABLE 1. NIF GENE PRODUCTS

| *nif* gene | $10^{-3}$ $M_r$ of gene product | gene function |
|---|---|---|
| Q | ? | ? |
| B | ? | FeMoco synthesis or processing |
| A | 57† | regulation |
| L | 45† | regulation |
| F | 17‡ | electron transport |
| M | 28§ | processing Kp2 |
| V | 42§ | processing Kp1 |
| S | 45‡ | ? |
| U | 32, 25§ | ? |
| X | 18§ | ? |
| N | 50‡ | FeMoco synthesis or processing |
| E | 46‡ | FeMoco synthesis or processing |
| Y | 21§ | regulation? |
| K | 60‡ | β-subunit of Kp1 |
| D | 56‡ | α-subunit of Kp1 |
| H | 35‡ | subunit of Kp2 |
| J | 120‡ | electron transport |

References for identification of gene products are: †, F. C. Cannon (unpublished); ‡, Roberts *et al.* (1978); §, Puhler & Klipp (1981).

## Nif *gene products*

The molecular masses and probable functions of most *nif* gene products are listed in table 1. *K. pneumoniae* nitrogenase is composed of two redox proteins (for review see Mortenson & Thorneley 1979). The Fe protein, also termed Kp2 ($M_r$ 68000) is a dimer composed of two identical subunits specified by *nif*H (Roberts *et al.* 1978). It contains a single $Fe_4S_4$ cluster and acts as an electron donor to the MoFe protein (Kp1) ($M_r$ 218000). Kp1 contains 2 Mo atoms and approximately 32 Fe atoms, some of which are present as $Fe_4S_4$ clusters. Kp1 is a tetramer of two non-identical subunits (α and β, $M_r$ 56000, 60000 respectively), which are the products of *nif*D (α) and *nif*K (β). The products of *nif*HDK alone cannot generate active nitrogenase and there is now considerable evidence that suggests post-translational processing or modification of the nascent polypeptides by other *nif* gene products.

A low molecular mass cofactor (FeMoco), which can be extracted from Kp1 and may contain the active site of the enzyme, partly restores activity to nitrogenase in cell extracts of *nif*B, *nif*E and *nif*N mutants (Roberts *et al.* 1978). These mutants are probably defective in the synthesis of FeMoco and/or its insertion into Kp1. Polypeptides with relative molecular masses of 46000 and 50000 have been assigned to *nif*E and *nif*N respectively (Roberts *et al.* 1978). Nitrogenase activity, which is impaired in *nif*M mutants, is partly restored in cell extracts of these mutants by the addition of purified Kp2, indicating that the product of *nif*M may play a role in the maturation of Kp2 (Roberts *et al.* 1978). The product of *nif*V is probably involved in processing Kp1, since purified Kp1 from a *nif*V mutant had altered substrate reduction properties (P. McLean & R. A. Dixon, unpublished).

The products of *nif*F ($M_r$ 17000) and probably *nif*J ($M_r$ 120000) are involved in the electron transport system for nitrogenase. The low levels of nitrogenase activities *in vivo* in *nif*F and *nif*J mutants are significantly enhanced in cell extracts assayed *in vitro* with sodium dithionite as an electron donor for nitrogenase (Roberts *et al.* 1978). Pyruvate can also act as an electron donor to nitrogenase in wild-type extracts but not in extracts of *nif*F and *nif*J mutants. Pyruvate-




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supported activity can be restored in extracts of *nif*F (but not *nif*J) mutants by the addition of *Azotobacter chroococcum* flavodoxin, which suggests that the product of *nif*F is an electron-transport protein (Hill & Kavanagh 1980; Nieva-Gomez *et al.* 1980).

The phenotypes of *nif*A and *nif*L mutants suggest that their products have regulatory functions. The product of *nif*A is required for the expression of all *nif* genes (except *nif*A and *nif*L) (Dixon *et al.* 1980; MacNeil *et al.* 1978; Roberts *et al.* 1978). The role of the *nif*A product as a positive activator of *nif* transcription is supported by the results of complementation tests in which the product of *nif*A can activate *nif* derepression in *trans* (Dixon *et al.* 1977; MacNeil *et al.* 1978). A specific regulatory function has not yet been assigned to the product of *nif*L, although there is evidence to suggest that it is involved in repression of the *nif* gene cluster (S. Hill, C. Kennedy & M. Merrick, unpublished). The *nif*Y gene product may also have a regulatory role since it appears to be required for full expression of the *nif*HDKY operon (M. C. Cannon, unpublished). The functions of the remaining genes, *nif*Q, S, U, X are unknown at present.

### *Regulation of* K. pneumoniae nif *genes*

Ammonia and other forms of fixed nitrogen, including nitrate and amino acids, prevent the expression of nitrogenase activity. Vigorous aeration also blocks nitrogenase expression (St John *et al.* 1974; Eady *et al.* 1978). $N_2$ is not required as an inducer of *nif* expression, since *nif* is expressed in cultures grown under argon (Parejko & Wilson 1970). Therefore the expression of *nif* is mediated by depression rather than an induction mechanism.

Regulatory studies suggest that *nif* derepression in limiting ammonia concentrations is initiated by a protein or proteins involved in glutamine synthetase (Gs) expression, which appears to act as a transcriptional activator of *nif* (Streicher *et al.* 1974; Ausubel 1979). The same regulatory protein(s) are required for the expression of several other operons, including *hut* (histidine utilization), involved in the assimilation of fixed nitrogen sources (Tyler 1978). At least one of these regulatory genes is closely linked to *gln*A, the structural gene for GS (Streicher *et al.* 1974; Ausubel *et al.* 1979; Pahel & Tyler 1979; Kustu *et al.* 1979; G. Espin & M. Merrick, unpublished).

A model for *nif* derepression that is consistent with the available evidence is that the *gln*-gene controlling protein(s) act at a single site in the *nif* cluster, i.e. the promoter of the *nif*LA operon, to activate *nif*A transcription. The product of *nif*A in turn activates transcription of the other *nif* operons. Results consistent with this model are briefly summarized as follows: (1) *nif*A mutations are pleiotropic on the other *nif* operons (Dixon *et al.* 1977; MacNeil *et al.* 1978; Roberts *et al.* 1978); (2) *gln*A-linked mutations can give rise to either a partial *nif* constitutive or a *Nif*$^-$ phenotype (Streicher *et al.* 1974; Tubb 1974; Ausubel *et al.* 1979; G. Espin & M. Merrick unpublished); (3) *cis*-dominant *nif* mutations have been isolated that are independent of *gln*-mediated regulation (Ausubel *et al.* 1977) (these mutations, called *nif*T, map in the *nif*L–A region (C. Kennedy, unpublished)); (4) although the product of *nif*A is required for the expression of the other *nif* operons it is not required for its own synthesis (Dixon *et al.* 1980; F. C. Cannon, unpublished); (5) gene fusions in which the *E. coli lac* operon was fused to individual *nif* promoters have been used to investigate derepression of *nif* operons independently of nitrogenase activity (Dixon *et al.* 1980). The results of these studies show that derepression of all *nif* operons is coordinate.

All identifiable *nif* gene products are absent in ammonia-grown cultures (Eady *et al.* 1978; Roberts *et al.* 1978) and *nif* mRNA is undetectable by DNA–RNA hybridization in repressed




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cultures (Janssen *et al.* 1980). *Nif–lac* fusions have also been used to show that all *nif* operons are repressed by ammonia, although the level of repression varies among different fusions (Dixon *et al.* 1980). When ammonia is added to a fully derepressed culture, a rapid repression of the Nif phenotype occurs (Tubb & Postgate 1973) and both nitrogenase activity and polypeptides disappear within 40 min (Collmer & Lamborg 1976). Studies on the repression of *nif*A and L gene clones suggest that the rapid repression of the *nif* operons by ammonia is not regulated by the removal of the *nif*A product (F. C. Cannon, unpublished). The results of these experiments show that although the rate of *nif*A and *nif*L polypeptide synthesis is proportional to the concentration of ammonia present in the culture during derepression, they are synthesized at ammonia concentrations that completely repress the nitrogenase structural gene operon. This suggests that rapid ammonia repression is mediated either through an attenuator mechanism or by an allosteric repressor. Repression studies with *nif*L mutants, which show that they are less sensitive to *nif* repression by $O_2$ (Dixon *et al.* 1980; S. Hill & C. Kennedy, unpublished) and fixed nitrogen (M. Merrick, unpublished), suggest that the *nif*L product has a repressor function. A model for ammonia repression of *nif* that is consistent with the evidence summarized above is that immediate repression of *nif* by the addition of ammonia to a derepressed culture is mediated through *nif*L product. The repression of GS by ammonia leads, in turn, to repression of the *nif*LA operon and thus concomitant removal of the *nif* repressor and activator. Repression of *nif* in ammonia-grown cultures would therefore be maintained solely by the absence of *nif*A product.

Studies with *nif*::*lac* fusions provide evidence that the nitrogenase structural gene operon in contrast to the other *nif* operons is autogenously regulated (Dixon *et al.* 1980). The presence of molybdate and product(s) of the *nif*HDKY operon is required for maximum expression of this operon. Although there is evidence to suggest that the *nif*Y gene product is required for maximum expression from the *nif*H promoter, it has not yet been assigned a specific regulatory function.

## Strategies

The possibilities for genetic manipulation in animal cell cultures, in yeast strains and in several bacterial genera have increased dramatically with the recent development of transformation procedures and of a variety of gene vectors appropriate to each system. Studies of foreign gene expression in these systems are no longer hindered by problems of exogenous DNA uptake and maintenance. The type of vector used for the introduction of these genes usually determines whether they are maintained on autonomously replicating plasmids or by chromosomal integration. Vectors used for yeast transformation provide some illustrative examples. An indigenous 2 μm yeast plasmid and a centromere fragment of chromosome 6 have been used in the construction of two distinct groups of gene vectors that replicate autonomously in *Saccharomyces cerevisiae* (Fink 1980). A third group of vectors that promote chromosomal integration is derived from *E. coli* gene vectors carrying yeast markers such as *leu*, *his* and *ura*. The latter provide homology with the chromosome of origin and usually determine the site of integration for linked genes.

Although efficient procedures for the transformation of plant cells have not yet been developed some success has been achieved by using cauliflower mosaic virus (CaMV) DNA and Ti plasmid DNA from *Agrobacterium tumefaciens* (Hohn *et al.* 1980; Hernalsteens *et al.* 1980; Cocking, this symposium). Both of these DNA molecules are potentially useful for vector con-




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struction. Vectors similar to those based on the 2 μm yeast plasmid could be derived from the CaMV DNA. Since the T-DNA region of the Ti plasmid is known to integrate into plant chromosomes, it is possible that a vector carrying this region could promote integration of linked genes. The origins of replication from mitochondrial, chloroplast and plant chromosomal DNA are also potentially useful starting materials for vector construction. Progress in the construction of vectors is seriously hindered at present by the lack of useful plant selectable markers.

Although the availability of gene vectors is a prerequisite for the transfer of *nif* genes to plant cells, developing mechanisms for their expression will probably be a more challenging problem to resolve because of this organizational and regulatory complexity. An example is the recent transfer without expression of *nif* to *S. cerevisiae*, which was achieved with clones of the entire *nif* gene cluster (Elmerich *et al.* 1980). If these clones were transferred to *Schizosaccharomyces pombe* it is possible that at least transcription of the regulatory *nif*LA operon would be activated by protein(s) involved in the expression of glutamine synthetase in this yeast that has characteristics similar to those of the *Klebsiella gln* system (Van Ardel & Brown 1977).

The location into which *nif* genes are manipulated in plant cells will determine the type of regulatory sequences required for their expression. A chromosomal location would probably require the fusion of each essential *nif* gene to a eukaryotic promoter. A promotor from the T-DNA region of the Ti plasmid would be potentially useful for this purpose. In chloroplasts, however, the expression of *nif* may not require such an extensive degree of manipulation, since chloroplast gene expression appears to be compatible with that of prokaryotic organisms. It may be that chloroplast RNA polymerase could initiate transcription at the *nif* promoters in the presence of *nif* activator protein.

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## *Discussion*

G. Pontecorvo, F.R.S. Would the energy consumption of nitrogen fixation not be deleterious in any new diazotrophic system?

J. R. Postgate. I thank Professor Pontecorvo for asking that question, because already in private discussion I have met several people who regard the ATP demand of nitrogen fixation as 'enormous' or 'crippling'. This is not true, so I think the point needs clarifying.

One can write a general equation for biological nitrogen fixation so:

$$N_2 + 3NADH + 5H^+ + 12ATP \longrightarrow 2NH_4^+ + 3NAD + 12ADP.$$

The 12 ATPs consumed by nitrogenase are the source of the oft-quoted 'huge energy demand' of nitrogenase because, on thermodynamic grounds, one would expect little or no energy loss in the overall reaction. To those 12 ATPs one must add 9 ATPs that could have been generated from 3 NADH (at a P/O ratio of 3) had they not been deflected into diazotrophy. Thus the basic cost of nitrogenase function becomes 10.5 ATP lost per N atom brought to the level of $NH_4^+$. I shall mention factors that alter that estimate later.

This is a crude estimate of the energy debt to an organism if it uses $N_2$ instead of $NH_4^+$. However, plants very rarely use $NH_4^+$; they use nitrate. One can write a corresponding general equation for nitrate reduction so:

$$NO_3^- + 4NADH + 6H^+ \longrightarrow NH_4^+ + 4NAD + 3H_2O.$$




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The consumption of 4 NADH brings the basic cost of nitrate reduction to 12 ATP lost per N atom brought to the level of $NH_4^+$.

The superficial impression that nitrate reduction is less efficient than diazotrophy is something of an illusion because these calculations exclude: (i) loss of $H_2$ by a side reaction of nitrogenase, which can add another ATP; (ii) ATP consumption in assimilation of $NH_3$, which is probably much the same for both processes; (iii) the energy cost of transporting $NO_3^-$ into the cells, which diazotrophy probably avoids; (iv) the fact that 12 ATP/$N_2$ is a minimum figure for consumption by nitrogen as nitrogenase; (v) the energy costs of synthesizing and maintaining nitrogenase compared with nitrate reductase; and (vi) the differential effects of the localization of the two processes in plants (e.g. in roots compared with leaves). Some of these factors will alter the energy budget substantially in practice, the trend being to favour nitrate reduction over diazotrophy, but the important message is that, a*s biochemical processes*, diazotrophy and nitrate reduction can be expected to make very similar demands on the plant's energy budget. This is indeed borne out in plant experiments (see, for example, Minchin & Pate 1973; Silsbury 1977), where the differences between nitrate-grown and $N_2$-grown legumes can be quite small.

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