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J. E. Beringer & P. R. Hirsch

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Genetic Engineering and Nitrogen Fixation

J. E. BERINGER AND P. R. HIRSCH

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, UK

Introduction

Nitrogen is extremely important in agriculture because it is a constituent of proteins, nucleic acids and other essential molecules in all organisms. Most of this nitrogen is derived from reduced or oxidized forms of N in the soil by growing plants, because plants and animals are unable to utilize N_2, which is abundant in the atmosphere. Under most cropping conditions N is limiting for growth and is provided in fertilizers, usually at rates of between 50 and 300 kg of N per ha per year (Anonymous, 1979). The only other sources available to plants are from decomposing organic matter, soil reserves, biological nitrogen fixation, the little that is deposited in rainfall and from other sources such as automobile exhausts.

Biological nitrogen fixation, the enzymic conversion of N_2 gas to ammonia, is much the most important source of fixed nitrogen entering those soils which receive less than about 5 kg N per ha per year from fertilizers. The reduction of N_2 is catalysed by the nitrogenase system, which is very similar in composition and function in all prokaryotes which produce it. Indeed, subunits of nitrogenase obtained from different nitrogen-fixing species can often be mixed to produce a functional system (Emerich and Burris, 1978). In addition, DNA coding for the structural proteins is so highly conserved in sequence that this coding has been used in hybridization experiments to demonstrate the presence of these genes in all nitrogen-fixing species of prokaryotes tested (Mazur, Rice and Haselkorn, 1980; Ruvkun and Ausubel, 1980). Nitrogenase is found only in prokaryotic micro-organisms and thus eukaryotes, such as plants, can benefit from N_2 fixation only if they interact with N_2-fixing species of micro-organism or obtain the fixed N after the death of the organisms.

Nitrogenase functions only under anaerobic conditions because it is irreversibly inactivated by oxygen. The fixation of N_2 requires large amounts of energy, about 30 moles of ATP per mole N_2 reduced (Hill, 1976; Schubert and Wolk, 1982), and thus can act as a major drain for energy produced by N_2-fixing micro-organisms. The requirement for an anaerobic environment and large amounts of energy presents problems to the micro-organisms that fix N_2 and

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to the geneticists who wish to extend the range of N_2-fixing organisms. Many micro-organisms fix N_2 anaerobically and thus avoid the oxygen problem. However, energy production from organic compounds is usually much more efficient when they are metabolized by oxidative phosphorylation. Thus, in general, nitrogen fixation under aerobic or microaerobic conditions should be more efficient, unless too much energy is lost in protecting the enzyme from oxygen or replacing oxygen-damaged proteins.

An important consequence of the large energy cost for biological nitrogen fixation is that the activity of nitrogenase needs to be regulated very carefully to ensure that only the required amount of fixed N is produced. We discuss the regulation of N_2 fixation in *Klebsiella pneumoniae* in some detail in this chapter because a full understanding of how nitrogenase is regulated will be necessary if the transfer of N_2 fixation genes (*nif*) into other species, or even plants, is to be beneficial to the recipient organism.

The preceding remarks about the energy requirement and oxygen stability of nitrogenase point to two of the most important problems that will be faced in transferring *nif* genes to new hosts. In this review we will discuss other potential problems and show how our knowledge of the genetics of nitrogen fixation might be exploited in future.

N_2-fixing organisms and associations

The ability to fix N_2 is found in a wide range of prokaryotic micro-organisms, some of which are listed in *Table 1*. Genetic studies showing that the structural genes are highly conserved in all known N_2-fixing species (Mazur, Rice and Haselkorn, 1980; Ruvkun and Ausubel, 1980) suggest that the *nif* genes must have evolved and then spread throughout virtually all groups of prokaryotic micro-organisms. The very great similarities in DNA sequence imply that nitrogenase probably evolved after most of the major groups of prokaryotes diverged and thus that the distribution of the genes occurred as a result of gene transfer. If the evolution had been convergent, one might expect to see similar proteins, but much less similarity in DNA sequence. If this evolutionary argument is correct there is good historical precedence for the potential for

Table 1. Nitrogen-fixing prokaryotes of particular interest for genetic manipulation.

Genus	Properties of interest
Azotobacter	Aerobic N_2 fixer; genetic work in progress.
Azospirillum	Commonly associated with plant roots; genetic work in progress.
Rhizobium	Nodulates some species of leguminous plants; genetic studies of fast-growing species well developed.
Klebsiella	Genetics of *nif* well understood, particularly after transfer to *Escherichia coli*.
Frankia	Nodulates a range of unrelated shrubby dicotyledonous plants; some species can be cultured.
Rhodospirillum, Rhodopseudomonas	Photosynthetic bacteria; genetic work in progress.
Anabaena, Nostoc	Form symbiotic associations with a range of plants, fungi etc.; photosynthetic, genetic work in progress.

expanding the range of N_2-fixing organisms in the future. However, it should be pointed out that no eukaryotic organism has yet been shown to code for nitrogenase and thus the barriers to transfer discussed in this chapter may indeed present very real obstacles to geneticists, who even now are mere amateurs in relation to the processes that have directed evolution.

It should be made clear also that the amount of N_2 fixed by micro-organisms in nature is related almost directly to their access to available sources of energy. Thus for free-living micro-organisms levels of N_2 fixation seldom approach 5 kg N per ha per year (*see* Beringer, 1983), although free-living blue-green algae (cyanobacteria) can fix larger amounts when adequate moisture is available because they are photosynthetic and thus are able to meet their own needs for photosynthetically produced organic compounds. In general, with the possible exception of blue-green algae in rice paddies, it is unlikely that gene manipulation to improve N_2 fixation in agriculture will be worth contemplating for free-living micro-organisms.

A number of N_2-fixing micro-organisms grow in the rhizosphere of a range of crop plants, particularly grasses (Dobereiner and Boddey, 1981) and, under these conditions where root exudates are fairly abundant, have the potential to fix quite large amounts of N_2. Indeed, rates as high as 100 kg N per ha per year have been reported (von Bülow and Dobereiner, 1975). Unfortunately, experience has shown that the amounts of N_2 fixed are relatively low (probably around 10–20 kg N per ha per year; *see* Beringer, 1983) and that only a small part of this is available directly to the plant. While the potential sources of energy in the rhizosphere are greater than those in the bulk of the soil it must be remembered that many other micro-organisms are attracted to the exudates and will also compete with the plant for fixed N released by the N_2-fixing species. Whether such associations can be exploited in the future to improve nutrient exchange, and hence the benefit to the plant, remains to be determined. Until we know much more about these associations it is difficult to see how important gene manipulation could be in improving associative N_2 fixation for crop production.

Much the most important N_2-fixing associations that occur in nature, both in terms of the amounts of N_2 fixed and the benefits to particular crops, involve symbiotic associations in which plants provide specific structures in which the micro-organisms are contained. The best-known examples are root nodules which are induced by species of *Rhizobium* on leguminous plants, by the actinomycete *Frankia* on a range of woody dicotyledonous plants, by the blue-green alga *Nostoc* on cycads, and the special pockets in the leaves of the water fern *Azolla* containing the blue-green alga *Anabaena*.

In all of these symbioses the micro-organisms live inside the plant as monocultures. They receive carbon compounds directly from the host and the fixed N is made available directly to the host. Indeed, it is becoming clear now that the micro-organisms are modified in the host so that when nitrogenase is functioning they are no longer able to assimilate the fixed N and thus they release NH_4^+ directly to be assimilated by the plant (O'Gara and Shanmugam, 1976; Peters *et al.*, 1982). Furthermore, the plant can regulate N_2 fixation by limiting the amount of infected tissue (e.g. the number of nodules) and by regulating carbon flow to the micro-organisms.

In relation to the comments made earlier in this chapter about the efficient use of carbon compounds, it is clear (particularly for *Rhizobium*, which is an aerobe) that N_2 fixation will be most efficient in a micro-aerobic environment. In the legume root nodule this is facilitated by producing haemoglobin which ensures that O_2 is transported to the centre of the nodule, which otherwise could become anaerobic, because of the large number of bacteria present (probably about 10^6–10^7 per nodule), at a sufficiently low P_{O_2} to prevent inactivation of nitrogenase.

Thus in our estimation the symbioses provide an example of the extent to which plants and microbes have had to adapt themselves in order for nitrogenase to function in a way to provide the maximum benefit to the host. We feel that it is important to bear this sophistication in mind when considering possible approaches towards the production of novel N_2-fixing organisms.

Genetics of free-living nitrogen fixation

Some of the earliest studies on the biochemistry of nitrogenase were made using enzyme isolated from the anaerobe *Clostridium pasteurianum*, and there has been much interest in aerobic nitrogen-fixing bacteria such as *Azotobacter*. However, the greatest advances in our understanding of biological nitrogen fixation have involved *Klebsiella pneumoniae*, which is closely related to the non-nitrogen-fixing bacterium *Escherichia coli*, the standard organism for bacterial geneticists. Methods for genetic analysis in *E. coli* could be applied to *K. pneumoniae* and genes could be transferred between the two species and expressed in either of the two organisms. 'Classical' genetics with mutants defective in nitrogen fixation (Nif^-) showed the *nif* genes of *K. pneumoniae* to be located on the chromosome, between genes for histidine biosynthesis (*his*) and shikimic acid uptake (*shiA*). The proximity of the *his* and *nif* regions was used by Dixon and Postgate (1972) to transfer these regions entire from a strain of *K. pneumoniae*, carrying a conjugative gene-mobilizing plasmid, to an *E. coli* strain which required histidine. *E. coli* recipients no longer requiring histidine for growth were found to have acquired the ability to fix nitrogen. Subsequently, a conjugative plasmid, pRDI, with a wide host-range and which had picked up the *K. pneumoniae nif* and *his* genes, was selected and transferred to several different bacterial genera (Dixon, Cannon and Kondorosi, 1976).

Complementation analysis of different *nif* mutations, transductional and other forms of genetic mapping, and the physical purification of DNA fragments by cloning into small plasmids have led to the construction of a physical map over a region of about 24 kb which is the *nif* DNA (Cannon, Riedel and Ausubel, 1979; Riedel, Ausubel and Cannon, 1979) and to the recognition and mapping of 17 separate genes within the *nif* cluster. Mutagenesis using DNA elements such as transposons and bacteriophage Mu also facilitated the identification of eight operons (groups of genes transcribed as a block from one promotor) within the *nif* gene cluster (Dixon *et al.*, 1977; Kennedy, 1977; MacNeil *et al.*, 1978; Merrick *et al.*, 1980; Pühler and Klipp, 1981).

More recently, RNA transcripts have been mapped and the promotor regions from the *nif* gene cluster have been cloned and sequenced so that the initiation

point for each operon is now known (Beynon *et al.*, 1983; Drummond *et al.*, 1983; Sundaresan *et al.*, 1983). One method used to identify promotors is to fuse a fragment lacking a promotor but carrying the structural gene for β-galactosidase (*lacZ*), to putative promotor regions. Promotor activity can then be measured by assaying β-galactosidase activity, and this has contributed towards understanding the control of expression of the *nif* operons (Dixon *et al.*, 1980; Hill *et al.*, 1981; MacNeil, Zhu and Brill, 1981; Merrick *et al.*, 1982; Drummond *et al.*, 1983; Ow and Ausubel, 1983). The genetic map, gene functions, organization and regulation are summarized in *Figures 1–3* and discussed below.

Nitrogenase has two soluble protein components, I and II. Component I consists of two copies of two subunits Iα and Iβ, which are encoded by *nifK* and *nifD* respectively. It contains both Mo and Fe atoms and is often referred to as the iron–molybdenum protein. Component II has two identical subunits encoded by *nifH*. Because it contains Fe atoms it is sometimes referred to as the Fe protein. These three genes, together with *nifY* (the function of the gene product is not yet known), are arranged in one operon. It is suggested that *nifF* encodes a flavodoxin which transfers electrons to nitrogenase, and that *nifJ* encodes a pyruvate oxidoreductase which transfers electrons from pyruvate to the flavodoxin. Products of two other genes, *nifM* and *nifS*, are required for processing the *nifH* gene product, component II of nitrogenase (Roberts *et al.*, 1978). Functional nitrogenase requires an inorganic cofactor containing Fe and Mo atoms, known as FeMoCo and four genes, *nifB*, *nifN*, *nifE* and *nifV*, are involved in its synthesis and maturation (Roberts *et al.*, 1978). Nitrogen fixation requires transfer of an electron from component II to component I with concomitant hydrolysis by component II of MgATP to MgADP. The main reductant for *in vivo* nitrogen fixation by *K. pneumoniae* is probably pyruvate, and *nifF* and *nifJ* gene products are required for transport of electrons from pyruvate via a flavodoxin to component II (Hill and Kavanagh, 1980). The functions of three other *nif* genes (*nifQ*, *nifU*, *nifX*) are not yet known.

All the *nif* operons (shown in *Figure 1*) are regulated by products of *nifA* and *nifL*. The *nifA* gene product is required for expression of all the *nif* operons except *nifLA*, and the *nifL* gene product represses expression, probably by inactivating the *nifA* gene product.

Nitrogenase is expressed only under certain physiological conditions: the enzyme is rapidly and irreversibly inactivated by exposure to oxygen and *K. pneumoniae* fixes nitrogen only in anaerobic or extremely micro-aerobic conditions. It is a facultative aerobe and when exposed to O_2, *nif* expression rapidly ceases (Eady *et al.*, 1978), probably because of *nifL*-mediated repression (Hill *et al.*, 1981). Nitrogen fixation uses energy, and when fixed nitrogen (nitrate, ammonia or amino acids) is available *nifA* expression is repressed, as are other genes involved in nitrogen metabolism. The regulation of genes under 'nitrogen control' is therefore of critical importance to expression of *nif*.

Early models for regulation of genes under nitrogen control in *K. pneumoniae* proposed that glutamine synthetase (GS) was responsible, but recent work with *E. coli*, *Salmonella typhimurium* and *K. pneumoniae* has shown them to be incorrect. Merrick (1982) has reviewed the evidence and presented a new model for nitrogen control in these bacteria. The structural gene for GS, *glnA* has

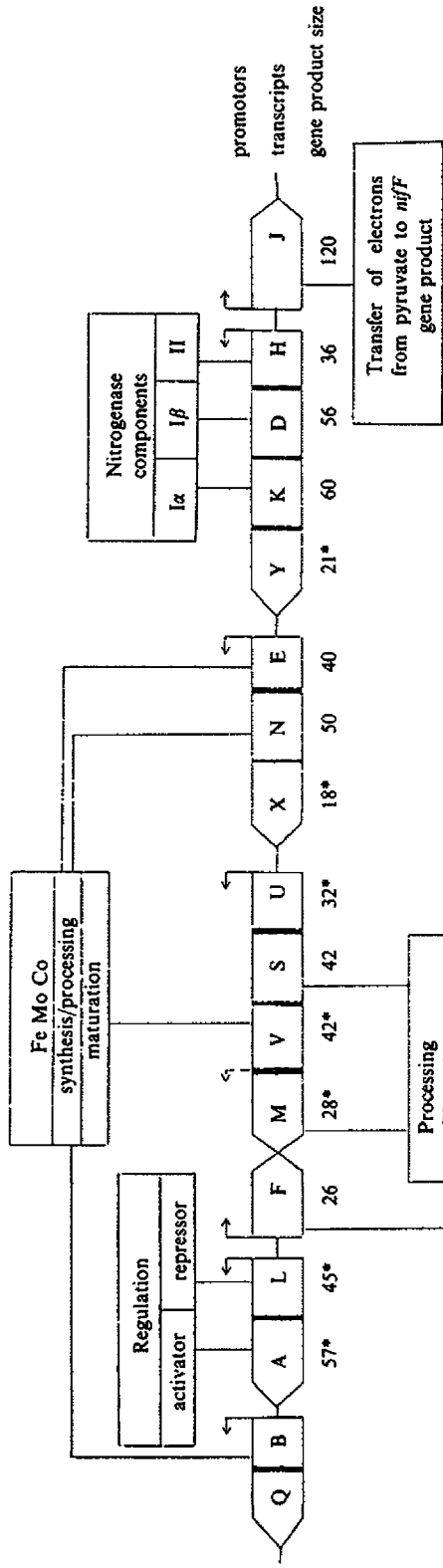

Figure 1. Summary of *nif* gene cluster organization, transcription and gene products. Transcripts and promotors according to Beynon *et al.* (1983) and M. Cannon (personal communication. *nifM* can also be transcribed off its own less efficient promotor. Gene product size: molecular weight $\times 10^{-3}$. * Ausubel and Cannon (1980), Roberts and Brill (1980).

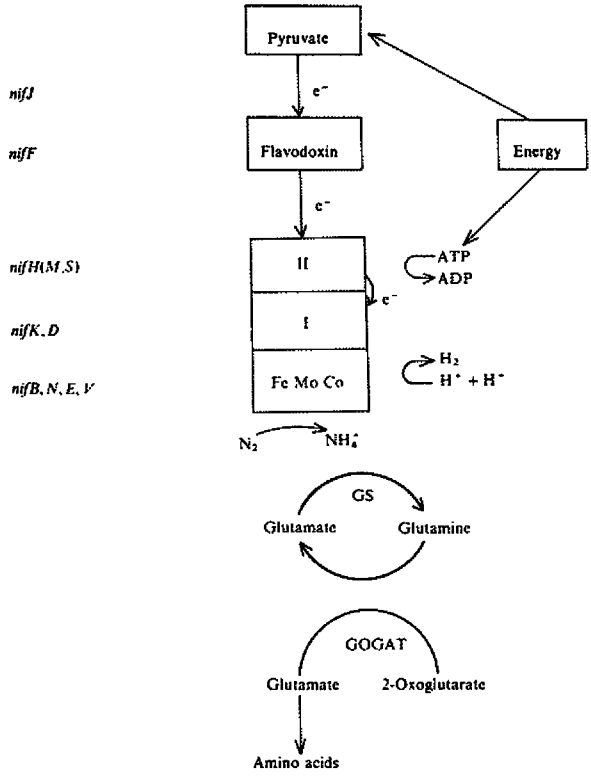

Figure 2. Functions of *nif* gene products in nitrogen fixation.

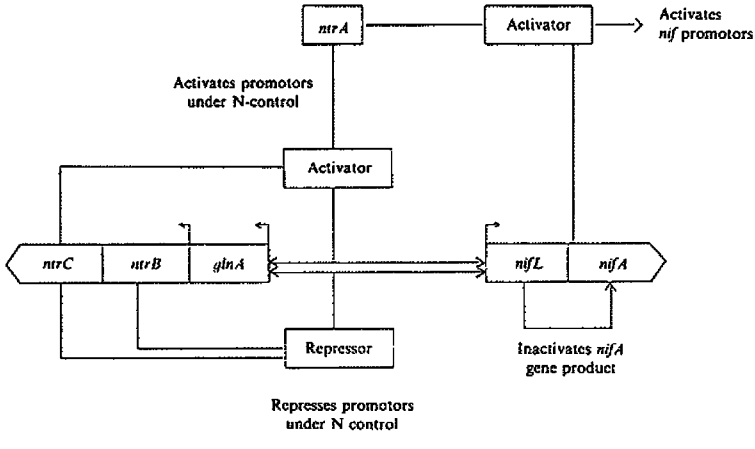

Figure 3. Nitrogen control and *nif* expression in *K. pneumoniae*.

been found to be the first in a transcriptional unit. The second gene, *ntrB* (formerly *glnL*) and the third, *ntrC* (*glnG*) are implicated in nitrogen control in conjunction with a separate gene, *ntrA* (*glnF*). Genes *ntrB* and *ntrC* also have their own weak promotor within the *glnA* operon. The *glnA* operon is transcribed constitutively at a low level, and the *ntrB* and *ntrC* gene products together repress operons under nitrogen control including *glnA* but not the internal *ntrB* promotor. This allows the constitutive expression of the *ntrB* and *ntrC* gene products. However, when the *ntrA* gene product is expressed, it associates with the *ntrC* gene product to form an activator for the *glnA* promotor and others under nitrogen control. It is proposed that *ntrC* encodes a DNA-binding protein which activates transcription in conjunction with the *ntrA* gene product, and that the *ntrB* gene product is either a competitor for DNA-binding sites with the *ntrC-ntrA* activator or inactivates it. In this model, (summarized in *Figure 3*), *ntrA* expression is essential for derepression of genes under nitrogen control (including the *nif* regulator *nifLA*) and must therefore be expressed when fixed nitrogen becomes deficient, in effect switching on the appropriate genes.

It has recently been reported (Drummond *et al.*, 1983; Ow and Ausubel, 1983) that the *nifA* gene product can substitute for *ntrC* in activating operons under nitrogen control in conjunction with the *ntrA* gene product. In addition, the *ntrC* or *nifA* gene products are necessary for transcription of the *nifLA* operon (Drummond *et al.*, 1983; Ow and Ausubel, 1983). The similarity between the *nifA* and *ntrC* gene products and the *nifLA* and *ntrBC* operons has led to the suggestion that *nifA* evolved from *ntrC* (Merrick *et al.*, 1982; Drummond *et al.*, 1983; Ow and Ausubel, 1983). However, they have diverged sufficiently to prevent *ntrC* from replacing *nifA* in activating the *nifH* promotor. The DNA sequences for all the *nif* operon promotors have recently been published (Beynon *et al.*, 1983; Drummond *et al.*, 1983) and have been shown to differ from other promotor sequences known in enteric bacteria. Beynon and co-workers (1983) report that the *nif* promotors contain similar DNA sequences arranged in two conserved regions. One of the regions is suggested to be specific for *nifA* protein binding, and the *nifLA* promotor alone has a slightly different sequence which may be because the primary activator is the *ntrC* rather than the *nifA* gene product. The other conserved region was found in all the *nif* promotors and may be specific to promotors expressed under conditions of nitrogen starvation. It is possible that the *ntrA* gene product interacts with RNA polymerase to produce a form which recognizes promotors of genes to be switched on under these conditions.

In summary, the *nifLA* operon is not transcribed when high levels of fixed nitrogen are present, but when this drops the *ntrA* gene product is active and in conjunction with the *ntrC* gene product de-represses transcription of *nifLA*. However, the *nifL* gene product prevents the *nifA* product from activating other *nif* promotors until the fixed-nitrogen level drops and nitrogen starvation begins. The *nifA* gene product is then active (the *nifL* product inactive) and, in association with the *ntrC* gene product, allows the *nif* operons to be expressed. Mutants with constitutive expression of *nifA* and no *nifL* gene products can transcribe the *nif* operons in the presence of fixed N and O_2 (Buchanan-Wollaston *et al.*, 1981; Merrick *et al.*, 1982). It is probable that the *nifL* gene product is in an

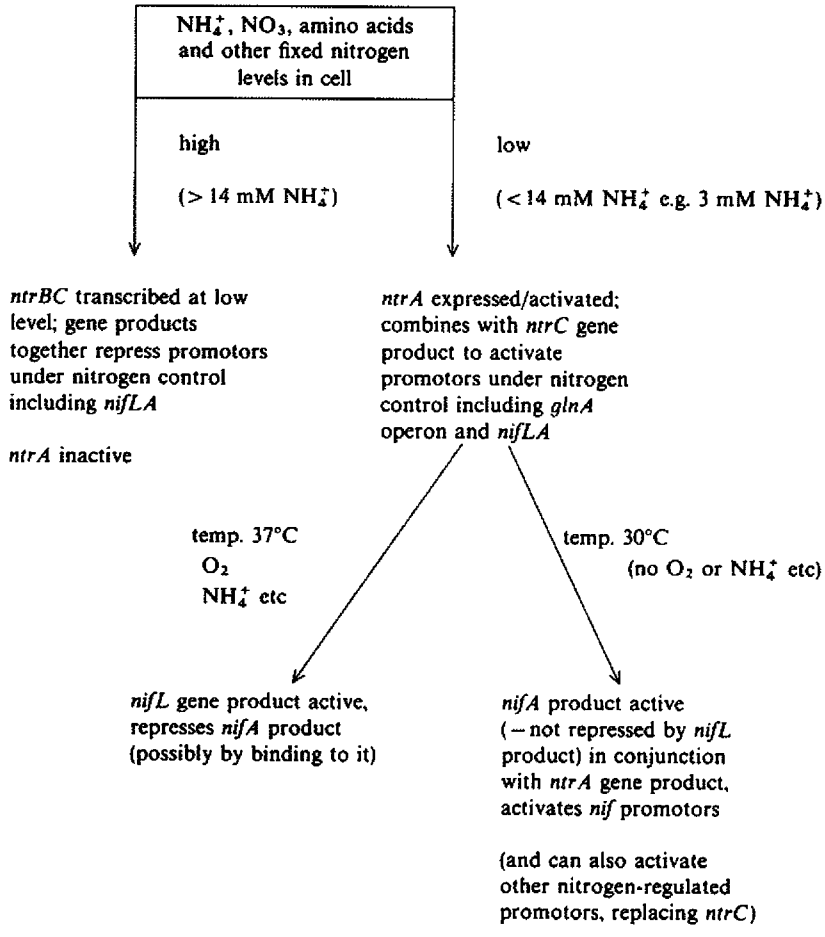

Figure 4. Regulation of *nif* expression in *K. pneumoniae* (after Merrick, 1982; Merrick *et al.*, 1982; Ow and Ausubel, 1983; Drummond *et al.*, 1983).

active form at high temperature or in the presence of fixed N and oxygen, but inactive at lower temperatures or in the absence of O_2 and fixed N (*Figure 4*).

Transfer of *nif* genes from *K. pneumoniae* to other organisms

The description of the *K. pneumoniae nif* genes and their regulation illuminates some of the problems which will be encountered when attempting to transfer *nif* to organisms other than the closely related enteric bacteria. Repression of *nif* expression by temperature, O_2 or fixed N can be overcome by deleting the *nifL* gene, which allows constitutive expression of the *nif* promotors mediated by the *nifA* and *ntrC* gene products. However, recognition of the *nif* promotors by DNA-dependent RNA polymerase is also required, and the ability of the host to translate the *nif* RNA correctly: ribosome-binding sites and codon usage

(i.e. which of the several possible triplet codons is generally used to code for a particular amino acid) must be compatible. For functional expression, still more factors are required. The nitrogenase must be protected from oxygen, but an energy source must be available to provide MgATP, a reductant (such as pyruvate) and a suitable electron-transfer system, for nitrogenase activity (although the *nifJ* and *F* gene products are implicated in electron transfer, other cellular components are certainly required). In addition, fixed N must be removed from the vicinity of the nitrogenase either by incorporation into amino acids or by excretion.

Nitrogenase activity in *E. coli* carrying the *nif* plasmid pRDI was found to be equivalent to that in the parent *K. pneumoniae* strain under suitable conditions (Dixon, Cannon and Kondorosi, 1976). Nitrogen fixation has also been reported in *Salmonella typhimurium* carrying *K. pneumoniae nif* genes (Cannon, Dixon and Postgate, 1976; Postgate and Krishnapillai, 1977). These enteric bacteria are all closely related and thus it is not surprising that expression was observed. Transfer of pRDI to *Agrobacterium tumefaciens* did not result in nitrogen-fixing recombinants. This species, which is an obligate aerobe, is not closely related to *Klebsiella* although it is a close relative of *Rhizobium* spp. which are symbiotic nitrogen-fixing bacteria. However, when *A. tumefaciens* carrying pRDI were grown with very low levels of O_2 and combined N, the presence of a protein cross-reacting antigenically to *K. pneumoniae* nitrogenase component I was detected (Dixon, Cannon and Kondorosi, 1976). These authors found no evidence for the expression of nitrogenase in *R. meliloti* carrying pRDI under similar conditions, but this species also has its own *nif* genes which are known to be repressed in free-living cultures. Mutants of *Azotobacter vinelandii* lacking either nitrogenase component I or II regained these activities when pRDI was transferred to them (Cannon and Postgate, 1976). This is significant because *Azotobacter* sp. are obligate aerobes which fix nitrogen in the presence of oxygen. The nitrogenase is thought to be protected from O_2 by intracellular compartmentalization and a very high respiration rate. A secondary mechanism to protect nitrogenase from oxygen inactivation is a protein which binds to the enzyme complex, first described by Shethna, Dervartanian and Beinert (1968) in *A. vinelandii*. Subsequently, purified protein was added to extracts from *Azotobacter* and shown to protect nitrogenase from oxidation. The O_2-protective proteins have been shown to contain Fe and S atoms and to bind to nitrogenase in the presence of O_2, preventing both N_2 fixation and irreversible O_2-inactivation (Robson, 1979). The O_2-protective protein of *A. vinelandii* has a molecular weight of 23000 (Shethna, Dervartanian and Beinert, 1968) and that of *A. chroococcum* is 14000 (Robson, 1979). It would be of great interest to isolate the genes which encode them, since it might be possible to transfer the O_2 protection to other nitrogen-fixing systems, and to new hosts together with the *K. pneumoniae nif* genes. However, it should be recognized that these proteins protect nitrogenase by binding to it and that the enzyme bound to these proteins is inactive. Thus N_2 fixation can re-start only when the oyxgen tension is sufficiently low to enable the proteins to be released without the enzyme becoming inactivated by oxygen.

The O_2 sensitivity of nitrogenase appears to be an intrinsic feature which

cannot be overcome by altering the proteins: the irreversible O_2 inactivation is due to the oxidation of non-haem Fe–S groups in both nitrogenase components (Robson, 1979). Another property of nitrogen fixation appears to be the evolution of hydrogen. Nitrogenase acts as a powerful reducing agent, reducing N_2 to NH_4^+, and the protons (H^+) which are abundant in living cells, to H_2. In the absence of N_2, H_2 is still evolved, with concomitant ATP hydrolysis. The proportion of N_2 fixed to H_2 evolved can vary (Andersen and Shanmugan, 1977). The process of N_2 fixation would require less energy if none were 'wasted' on H_2 evolution, but the two functions may be inseparable. However, some energy can be saved by recycling H_2 to H_2O, releasing electrons, and many N_2-fixing bacteria have 'uptake hydrogenases' (Evans *et al.*, 1981). It would therefore seem to be an advantage to introduce an uptake hydrogenase together with *nif* genes into new hosts which do not possess this function.

Any attempts to get *nif* genes expressed in eukaryotic cells will have to overcome considerable problems. There are basic differences in the organization, transcription and translation of genes in prokaryotes (i.e. bacteria) and the nuclei of eukaryotes (higher organisms). For instance, the promotor sequences recognized by the DNA-dependent RNA polymerases are different, as are the sites on the mRNA to which ribosomes bind and where translation is initiated. Furthermore, many bacteria have several genes transcribed from one promotor (as in the *nif* operons) resulting in polycistronic mRNA which has internal ribosome-binding sites for each gene, whereas eukaryotes almost invariably have monocistronic mRNAs with a binding site at the 5′ end. No internal ribosome-binding or re-initiation of protein synthesis occurs, unlike prokaryotes which can re-initiate translation within the mRNA (Kozak, 1983). Therefore, if *nif* were to be expressed as nuclear genes in a eukaryote, it would be necessary to fuse the coding sequence of each gene to eukaryotic promotors in such a way as to produce a suitable ribosome-binding site at the 5′ end of the transcript. Another potential problem with eukaryotic DNA is the 'switching off' of gene expression due to methylation of the DNA. This has been observed in some plant tumour tissues carrying integrated T-DNA from *A. tumefaciens* (Clarke, Pearson and Hepburn, 1983; Hepburn *et al.*, 1983).

It is perhaps surprising that expression of some prokaryotic genes has been reported in a eukaryote, the yeast *Saccharomyces cerevisiae*. These were antibiotic resistance genes derived from bacterial plasmids which are expressed in a wide variety of prokaryotic backgrounds. However, it was not proved that the genes (β-lactamase (Hollenberg, 1982); chloramphenicol acetyltransferase (CAT) (Cohen *et al.*, 1980); and neomycin phosphotransferase (NPT) (Jiminez and Davies, 1980)) were transcribed from their own promotors. Some β-lactamase has also been detected in the fungus *Podospora anserina* carrying a plasmid with a β-lactamase gene (Stahl *et al.*, 1982). No expression of CAT or NPT was detected in the fungus *Neurospora crassa* (Hughes *et al.*, 1983). To get expression of CAT and NPT in plant cells using the T-DNA as a vector, it was necessary to fuse the coding sequence to a eukaryotic promotor (Bevan, Flavell and Chilton, 1983; Herrera-Estrella *et al.*, 1983).

Although the *nif* gene cluster from *K. pneumoniae* has been transferred to *S. cerevisiae* (Elmerich *et al.*, 1981; Zamir *et al.*, 1981), expression of the genes has

not been reported, and it is highly unlikely that they could be transcribed correctly.

The organization of the *nif* genes in operons with a common regulator in *K. pneumoniae* is probably important. This will result in the required amount of each product being synthesized at the correct time. There may be slight differences between the amount of the products from the first and last gene in an operon; the extra promotor observed in front of *nifM*, the last gene in the *nifUSVM* operon (Beynon *et al.*, 1983), may be relevant in this respect. Co-ordinated regulation of *nif* expression would be necessary in a new host. For instance, the *nifM* and *nifS* products are required to process the *nifH* product, and equal amounts of nitrogenase components I and II must be present for efficient N_2 fixation. For example, an excess of component I results in more ATP hydrolysed by component II for each electron transferred to I (Ljones and Burris, 1972). Normally two ATP molecules are hydrolysed for each electron transferred, and in *K. pneumoniae* 29 ATP molecules (i.e. 58 electrons) are required for each molecule of N_2 fixed (Hill, 1976); excess component I would therefore lead to ATP (and energy) being wasted during N_2 fixation.

Cloning, in the field of molecular biology, has come to mean the isolation and propagation of individual DNA fragments (often encoding a specific gene) by insertion *in vitro* into small plasmid vectors which can be transformed into, and selected for, in *E. coli*. For successful transformation certain factors are required. First, the organisms in question must be able to take up DNA: many animal cells can take up exogenous DNA, and this has been achieved with some fungi and plants by incubating protoplasts with DNA and then allowing regeneration of the cell wall. Fusion of protoplasts with artificial liposomes containing DNA has also been successful (Fraley and Papahadjopoulos, 1982). Other methods include micro-injection, which has been used to introduce DNA into *Drosophila* embryos (Germeraad, 1976) and the potential use of naturally infectious molecules such as the cauliflower mosaic virus (Hohn, Richards and Lebeurier, 1982). The phytopathogenic bacterium *A. tumefaciens* carries a large Ti (tumour inducing) plasmid, part of which becomes integrated in infected plant cells, and is known as the T-DNA. The mechanism of transformation is not yet understood and normally involves an interaction between the bacterium and plant cell; purified Ti plasmid DNA is also infective (Davey *et al.*, 1980; Krens *et al.*, 1982). The T-DNA can be used as a vector to introduce other genes into plant chromosomal DNA if the genes have been introduced previously into T-DNA which can be transferred to the plant by *Agrobacterium*. There are certain limitations to the system: (1) only certain dicotyledonous plants can be transformed; (2) the T-DNA produces a highly abnormal tumorous phenotype due to altered phytohormone levels; (3) deletions in the T-DNA are common. New vectors derived from T-DNA are being developed which produce apparently normal cells that can develop into plants capable of setting viable seed containing the transferred DNA. Some of these problems and advances are discussed by Cocking *et al.* (1981), Chilton (1983) and Schell and Van Montagu (1983). One disadvantage is that without the tumorous phenotype it is difficult to detect transformants. In bacterial and fungal transformation systems, the vector carries genes for enzyme activities which the host lacks: either

the gene replaces a defective one in host auxotrophic mutants, or specifies a new property such as antibiotic resistance. In such systems the presence of the vector can be maintained by constant selection for the genetic marker. The third requirement is the ability of the vector either to replicate autonomously in the host cell or to integrate into the host genomic DNA. In the former case it may be possible to have many copies of the vector; in the latter, only one or two (unless integration into a multiple-copy target site is possible).

Integration can be directed into specific sites if the vector carries a chromosomal DNA segment giving a region of homology. Such vectors can be made readily by introducing cloned host DNA into the vector. If the cloned DNA is for a known gene the site of integration of DNA cloned on the vector will be in or beside that gene in the host genome. To maintain genes carried on an autonomously replicating vector it is necessary to have a selectable marker. In the absence of selection, autonomously replicating plasmids may be lost rapidly as the host cells divide, and especially during meiosis. This problem may be overcome by cloning a chromosomal centromere into the plasmid: in *S. cerevisiae* such meiotically stable vectors have been constructed (Clarke and Carbon, 1980).

The organelles of eukaryotes are possible sites for the integration of foreign DNA. They resemble prokaryotes in some respects of their DNA organization: they contain their own RNA and protein-synthetic systems and their DNA genomes are circular, like bacterial chromosomes and plasmids. Plant mitochondrial genomes investigated so far are larger and more complex than those of animals (which are about 15 kb), ranging from 330 kb to 2400 kb (Ward, Anderson and Bendich, 1981) and smaller circular DNA molecules have been observed in some species. The ribosomes in plant mitochondria have features in common with both prokaryotic and eukaryotic cytoplasmic ribosomes (Levings, 1983). No promotor sequences for plant mitochondrial genes have been described, to our knowledge. The genome sizes of chloroplasts which have been reported ranged from 120 to 180 kb (Palmer, Singh and Pillay, 1983; Spielman, Oritz and Stutz, 1983) and all have an unusual structure with inverted repeats that can recombine to form two alternative orientations of the unique regions (Palmer, 1983).

The chloroplast genes for the large subunit of ribulose biphosphate carboxylase from maize and wheat have been shown to be transcribed and translated in *E. coli* and have prokaryotic-type promotor sequences (Gatenby, Castleton and Saul, 1981). It is therefore possible that the chloroplast transcription and translation system could allow the expression of prokaryotic DNA in plants. To expore this possibility it would be necessary to develop a transformation system to incorporate foreign DNA sequences into the chloroplast.

Transformation systems used successfully in *S. cerevisiae*, *N. crassa* and *P. anserina* could be relevant. The *S. cerevisiae* 2-micron DNA, a high copy number (50 copies per haploid genome) autonomously replicating circular plasmid, is the basis for many 'shuttle' vectors which can replicate in both *S. cerevisiae* and *E. coli* since they carry replication origins and gene-encoded functions which can be selected in either organism. The 2-micron DNA has an inverted repeat

which recombines at specific sequences (a function in which a 2-micron DNA encoded product is involved), and shuttle vectors carrying one inverted repeat and transformed into *S. cerevisiae* are often observed to recombine with the 2-micron DNA plasmid (Broach, 1982). This interaction is much more frequent than that attributable to normal homologous recombination. The interconversion of chloroplast DNA mediated by inverted repeats (reported by Palmer, 1983) could be caused by a similar mechanism, and it would facilitate the integration of foreign genes on vectors carrying one repeat into the chloroplast genome. An *N. crassa*, *E. coli* shuttle vector carrying part of an *N. crassa* mitochondrial plasmid which could replicate autonomously in both the mitochondria and cytoplasm of the fungus was observed to integrate into the mitochondrial DNA (Stohl and Lambowiz, 1983). Similarly, a hybrid plasmid carrying a mitochondrial replicon from *Podospora anserina* could replicate autonomously in *P. anserina* (Stahl *et al.*, 1982).

If mitochondrial replication origins support replication of hybrid plasmids in the cytoplasm or mitochondria of fungi, it is feasible that organelle DNA origins could support replication of hybrid plasmids in plant cells. Since transformation systems for fungi (described above) and dicotyledonous plants using T-DNA (*see* next section) are now available, the feasibility of directed integration into organelle DNA or autonomous replicating vectors located within organelles could be tested. However, it is necessary to recall the constraints on *nif* expression: even if the promotors could be transcribed and the RNA translated, the enzymes would not function unless the physiological requirements were met. One problem with chloroplasts is that during photosynthesis, oxygen is evolved which would inactivate the nitrogenase, so no N fixation would be possible in the light, even if an O_2-protection system were provided (such as the *Azotobacter* protein described previously). However, during darkness the conditions would be more suitable.

Symbiotic N_2 fixation (*rhizobium* and legumes)

The most important N_2-fixing symbiosis is that of *Rhizobium* and leguminous plants, in which the bacteria fix nitrogen in root nodules. The association is rather specific: only certain *Rhizobium* strains can nodulate a particular host plant, and this has led to the definition of *Rhizobium* 'species' according to the host plants which they can nodulate. For example, *R. trifolii* nodulates *Trifolium* sp.; *R. phaseoli* nodulates *Phaseolus* sp.; *R. meliloti* nodulates *Melilotus* sp.; *R. leguminosarum* nodulates several legume spp. including *Pisum*, *Lathyrus*, *Lens* and *Vicia*, and *R. japonicum* nodulates *Glycine*.

The rhizobia include two distinct subgroups which can be classified according to their behaviour in culture as 'fast' or 'slow' growers. The slow-growing rhizobia have been shown to be able to fix N_2 *in vitro* under micro-aerobic conditions with trace amounts of fixed N and a carbon compound such as succinate for an energy source (Kurz and LaRue, 1975; McComb, Elliot and Dilworth, 1975; Pagan *et al.*, 1975). However, partly because of their growth characteristics, very little genetic analysis has been possible with the slow growers. In contrast, great progress has been made with genetic analysis in the fast growers:

however, no conditions under which they can fix N_2 outside the host have been found, although recently a relatively fast-growing strain of *Rhizobium* that nodulates *Sesbania* has been shown to fix N_2 in pure culture (Dreyfus, Elmerich and Dommergues, 1983).

Although nitrogenase is O_2 sensitive, rhizobia are obligate aerobes and require some O_2 to fix N_2. Nodules containing N_2-fixing bacteroids contain large amounts of haemoglobin which, like animal haemoglobins, is involved in oxygen transport. The globin moiety is encoded by plant genes (Dilworth, 1969; Sullivan *et al.*, 1981) and synthesis appears to be regulated by the rhizobia, since it is induced only when rhizobia are present (Verma and Long, 1983). The haem moiety is probably synthesized by the rhizobia and exported into the cytoplasm (Cutting and Schulman, 1969; Dilworth, 1969).

The formation of the N_2-fixing symbiosis is complex and many genes from both plant and bacterium must be involved. Only a few plant genes involved in the establishment of the symbiosis have been identified and the functions of these are not clear (*see* Verma and Long, 1983).

RHIZOBIUM GENETICS

Auxotrophic and antibiotic-resistant mutants were isolated in *R. leguminosarum* and wide host-range antibiotic-resistant plasmids of the IncP1 group were found to be able to mobilize genes between different strains (Beringer and Hopwood, 1974) and species of *Rhizobium* (Johnston and Beringer, 1977). Subsequently similar circular linkage maps of chromosomal genes in *R. meliloti* (Kondorosi *et al.*, 1977; Meade and Signer, 1977) and *R. leguminosarum* (Beringer, Hoggan and Johnston, 1978) have been constructed. Chromosomal genes in *R. leguminosarum*, *R. trifolii* and *R. phaseoli* were found to be interchangeable (Johnston and Beringer, 1977) implying that nodulation specificity might be extrachromosomal, although mutations resulting in no nodulation (Nod^-) or no N_2 fixation (Fix^-) have been mapped to the chromosome (Beringer, Brewin and Johnston, 1980; Forrai *et al.*, 1983).

The host specificity of nodulation is now known to be plasmid-determined in *R. leguminosarum*, *R. phaseoli* and *R. trifolii*. A conjugative plasmid was identified in *R. leguminosarum* (Hirsch, 1979) and subsequently shown to carry genes specifying both nodulation of *Pisum*, and nitrogen fixation (Johnston *et al.*, 1978). This plasmid, pRL1JI, is about 200 kb in size (Hirsch *et al.*, 1980). A kanamycin-resistant derivative, pJB5JI, has been made by introducing the transposon Tn*5* into pRL1JI (Johnston *et al.*, 1978). It has facilitated transfer to many different *Rhizobium* sp., converting Fix^- *R. leguminosarum* mutants to Fix^+ and enabling *R. trifolii* and *R. phaseoli* to nodulate *Pisum* (Johnston *et al.*, 1978). A conjugative plasmid carrying *Trifolium* nodulation and N_2-fixation genes has been found in an *R. trifolii* strain which on transfer to *Agrobacterium tumefaciens* conferred the ability to nodulate clovers, although no N_2 fixation was detected (Hooykaas *et al.*, 1981), and an *A. tumefaciens* strain carrying pJB5JI has been shown to nodulate *Vicia hirsuta* (Van Brussel *et al.*, 1982). These experiments imply that many of the genes involved in nodulation and N_2 fixation are plasmid-encoded. Indirect evidence for the location of symbiotic

genes on plasmids has been obtained from Nod$^-$ mutants found to have plasmid deletions or to have lost plasmids (e.g. Beynon, Beringer and Johnston, 1980; Hirsch *et al.*, 1980; Zurkowski, 1982).

The homology observed between nitrogenase genes from different species has been used to look for *nif* genes in *Rhizobium*. They have been reported to be plasmid-borne in *R. leguminosarum*, *R. phaseoli* and *R. trifolii* (Nuti *et al.*, 1979; Hombrecher, Brewin and Johnston, 1981; Prakash, Schilperoort and Nuti, 1981), *R. meliloti* (Banfalvi *et al.*, 1981) and some fast-growing rhizobia which nodulate soybeans (Masterson, Russell and Atherly, 1982). In some cases the plasmids were very large, greater than 500 kb (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1982), and could be detected physically only by lysing bacteria *in situ* on agarose gels, separating plasmid DNA from the cellular debris and linear fragmented chromosomal DNA by electrophoresis. When transferred from the gel to a nitrocellulose filter, by 'Southern blotting', homologous DNA sequences on the plasmid can be detected by hybridization to a radioactive DNA probe. It is possible that in cases where *nif* homology could be detected in total DNA but not in the plasmid fractions, e.g. some *R. japonicum* strains described by Masterson, Russell and Atherly (1982), a very large plasmid was involved, or that these genes are carried in the chromosome of some species and strains.

Identification and mapping of genes involved in both nodulation and N_2 fixation has been facilitated by the technique of transposon mutagenesis using the transposon Tn*5*, which specifies kanamycin resistance, a convenient selectable marker for most *Rhizobium* strains. An unstable 'suicide' vector, pJB4JI, has been constructed to transfer Tn*5* to *Rhizobium* from *E. coli* (Beringer *et al.*, 1978): it consists of an IncP1 plasmid, Tn5, and the bacteriophage Mu which renders IncP1 plasmid replication unstable in many *Rhizobium* strains. *Rhizobium* exconjugants selected for kanamycin resistance are stable when Tn*5* has transposed from the unstable vector to chromosomal or plasmid DNA, and since Tn*5* appears to transpose randomly to *Rhizobium* DNA a great number of different mutants can be obtained. The Tn*5* mutations can be mapped genetically, by transduction, conjugation (Beringer *et al.*, 1978) and physically, using Tn*5*-DNA probes (Dhaese *et al.*, 1979). Any Tn*5* mutations can be isolated in *E. coli* by digesting DNA with a restriction endonuclease that does not cut internal sites in Tn*5*, cloning into a small plasmid, and selecting *E. coli* transformants. Such clones will contain the regions of the target gene which border the Tn*5* insertion. These can be mapped for restriction sites, and also used to detect *E. coli* transformants carrying the intact gene cloned from unmutated *Rhizobium* (usually from a 'library' cloned into cosmids, which are vectors that select for the insertion of large DNA fragments). The cloned gene can then be transferred back to *Rhizobium* mutants to check phenotypic complementation (Scott *et al.*, 1982). Other methods have utilized the homology of *K. pneumoniae* and *Rhizobium nif* genes to select for transformants carrying cloned *Rhizobium nif* DNA. This method has facilitated a molecular analysis of the *nif* genes of *R. japonicum* which was not possible using 'classical' genetics. Using defined Tn*5* insertions selected in *E. coli*, mutated genes could be transferred back to *Rhizobium* and recombined into the homologous DNA, causing 'reverse mutagenesis' (Ruvkun and Ausubel, 1981).

Using such methods, the organization of the plasmid regions carrying Nod and Fix genes in *R. meliloti* (Ruvkun, Sundaresan and Ausubel, 1982) and *R. leguminosarum* (Ma *et al.*, 1982; Downie *et al.*, 1983) have been shown to be similar. Two regions in which Fix^- mutations map are separated by a relatively long section, within which Nod^- mutations map. In *R. leguminosarum*, a region with *nifHD* homology (in which Fix^- mutants map) is separated by 20 kb from the region in which Nod^- mutants map; a further 6 kb from this is the second Fix region, which has homology with *K. pneumoniae nifA* DNA (Downie *et al.*, 1983). This was similar to the arrangement in *R. meliloti* (Long, Buikema and Ausubel, 1982). Site-directed mutagenesis in the *nifHDK* region of *R. meliloti* has shown that the direction of transcription is the same as that in *K. pneumoniae* and that the three genes are transcribed from one promotor (Ruvkun, Sundaresan and Ausubel, 1982). This implies that some aspects of the organization of the two *nif* regions are conserved (although the *nifK* gene in *Rhizobium* is sufficiently different from its *K. pneumoniae* counterpart not to show DNA sequence homology). The *nifHDK* genes from a slow-growing *R. japonicum* strain have been cloned (Hennecke, 1981) and shown to be arranged and transcribed in the same direction as a single unit, as in *K. pneumoniae* (Fuhrmann and Hennecke, 1982).

The report that the *nifHDK* promotor from *R. meliloti* is activated in *K. pneumoniae* by the *nifA* gene product (Sundaresan *et al.*, 1983) is especially interesting, since little is understood about the regulation of *nif* in *Rhizobium*. It does not seem to be inhibited in bacteroids exposed to 7·5 mM NH_4^+ (Scott, Hennecke and Lim, 1979), a level which would repress *K. pneumoniae nif* (due to activation of the repressor, the *nifL* gene product). This may imply that although a *nifA*-like product is involved in activation of transcription, it is itself not controlled by a *nifL*-like gene.

The methodologies of molecular biology have greatly increased our knowledge of the number and location of some of the bacterial genes involved in the *Rhizobium*–legume symbiosis, but there are still many unknown factors. The recognition between compatible bacteria and host plant, the steps leading to infection and bacteroid formation, and the induction of nitrogenase synthesis in the bacteroid and leghaemoglobin in the host, are not understood. Bacterial exopolysaccharides may be important in the first step (Chakravorty *et al.*, 1982), and hormone synthesis by rhizobia in the infection process and induction of meristematic activity in the root cortex (*see* Beringer *et al.*, 1979). Since nitrogenase synthesis does not appear to be repressed by fixed N_2, understanding the mechanisms by which it is induced is important.

The potential for transferring the ability to be nodulated from legumes to other plant groups is extremely limited, because we understand very little of the plant factors involved. Some improvements may be made to the rhizobia, but this may be limited to recognizing strains which are particularly good at fixing N_2 in a certain host, and inoculating the seed before planting. Provided that conditions are suitable for infection, and the inoculant strain can outcompete native rhizobia in the soil, great improvements in crop yield and seed quality are possible. It is possible to manipulate the rhizobia genetically, although what improvements could be made are not clear. The only factor so

far which appears to vary among strains is the presence of, or absence of, an uptake hydrogenase (Evans *et al.*, 1981, 1982) and genes for this have been transferred to strains which lack it (Brewin *et al.*, 1980) but it is not clear whether the efficiency (i.e. amount of fixed carbon consumed per molecule of N_2 fixed) is improved.

Future research with *Rhizobium* will reveal more information about the processes involved, and will certainly present new opportunities for genetic manipulation of strains, once the techniques for cloning and transfer of genes between strains have been fully developed.

Conclusions

Our knowledge of biological nitrogen fixation has advanced rapidly over the last few years, partly because of the amount of time and money invested in research, but largely because of the rapid advances in genetic methodology that have occurred in this time. For example, our understanding of the number, function and regulation of the *nif* genes of *Klebsiella pneumoniae*, which is described in this review, has depended largely upon the use of procedures to clone DNA, to move it into genetically different hosts and to study it and gene expression *in vitro*. That so much remains to be learnt is not so much a criticism of the research being done as a reflection of the great complexity involved in the production, functioning and especially the regulation of nitrogenase.

We have discussed briefly the concept of moving *nif* genes into new hosts to widen the range of nitrogen-fixing species in agriculture. That it will be possible to move these genes into plants which are susceptible to *Agrobacterium* is not in doubt; indeed it will probably be done during 1984. However, as stated previously, for expression to occur, promotor and other regulatory regions for these genes will need to be replaced with appropriate eukaryotic DNA sequences; in addition, some methods will be needed to regulate the *nif* genes to prevent the hosts being damaged or killed by excess ammonia and to ensure that energy is used efficiently. Whether sites can be exploited in which oxygen damage can be avoided remains to be demonstrated. While we feel that efficient nitrogen-fixing plants are unlikely to be produced in the near future, it is a tribute to modern genetics that we can consider their production as a rational goal in research on biological nitrogen fixation.

However, in striving to widen the range of species that are not dependent on fixed nitrogen, we should recognize that animals are major consumers of protein feeds, which in many cases could be used for human nutrition. Most domestic animals, particularly ruminants, have digestive systems which are often anaerobic and contain extremely large numbers of micro-organisms. There would appear to be enormous potential in producing nitrogen-fixing organisms to colonize these animals so that poorer quality feed containing much less protein (e.g. straw) could be utilized effectively. Undoubtedly there would be serious problems in producing micro-organisms which could colonize animal digestive systems and real problems in introducing *nif* genes into such micro-organisms. However, would these problems be as great as those which we expect to surmount in introducing *nif* genes into plants?

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