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Biological nitrogen fixation: fundamentals

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The enzyme responsible for N_2 fixation, nitrogenase, is only found in prokaryotes. It consists of two metalloproteins, both irreversibly destroyed by exposure to the O_2 of air. The MoFe-protein binds N_2 and the Fe-protein, after activation by MgATP, supplies electrons. H_2 is evolved during the reduction of N_2 to NH_3 and can become the sole reaction in the absence of N_2; valuable information has been obtained by exploiting the ability of nitrogenase to reduce substrates such as acetylene, azides and cyanides. Substrate quantities of MgATP are required for all such reactions.

The sensitivity of nitrogenase to oxygen is an important physiological constraint on its use and distribution; the ATP requirement and metal contents are less serious constraints. O_2 and NH_3 regulate synthesis and sometimes function of nitrogenase.

Nitrogen fixation by *Klebsiella pneumoniae* is genetically encoded by 17 genes (the *nif* genes) in a cluster of seven or eight operons. The functions of several of these genes are known and the outlines of their regulation can be discerned. The *nif* cluster can be transferred to new prokaryotic genera, sometimes yielding new diazotrophic strains or species; they have been transferred to yeast and are silent. They have been cloned and alien DNA (*lac*) has been fused into *nif*. Transfer of expressible *nif* to new genetic backgrounds has probably occurred in Nature and may be exploitable for agriculture.

For the purposes of this Discussion Meeting I divide this topic into two areas: the fundamentals of the N_2 fixation process itself and the fundamentals of ancillary processes that are either necessary or specially helpful to N_2 fixation (e.g. symbiosis, hydrogenase). Both areas are very broad and have been the subject of spectacular advances over the last decade or two (see recent symposia edited by Newton & Orme-Johnson (1980), Stewart & Gallon (1980), Chatt *et al.* (1980) and Gibson & Newton (1981)). In this contribution I shall restrict my coverage to information that has some bearing on N_2 fixation in the nitrogen cycle; documentation will be supplementary to that available from the published symposia just cited.

Nitrogen fixation

Microbiology of N_2 *fixation*

The agents of N_2 fixation must still be regarded as exlusively prokaryotic because the most recent claim for fixation by a eukaryote (Yamada & Sakaguchi 1980) has not been confirmed in another laboratory. A table of prokaryotic genera within which diazotrophic strains have been described forms table 1; a more detailed table, with a commentary, was presented by Postgate (1981). For present purposes I emphasize three points.

1. That diazotrophy is rarely universal within the genera listed and is often not universal even within a given species of a genus.

2. That most types earlier regarded as essentially symbiotic (e.g. *Rhizobium*, *Frankia*) are capable of diazotrophy *ex planta*, albeit in rather restricted environmental conditions, yet a few apparently obligately symbiotic diazotrophs still exist (e.g. most strains of *Rhizobium meliloti* or *R. leguminosarum*).

3. That the uncertain diazotrophic status of the genus *Pseudomonas* derives from the fact that, of the two seemingly authentic strains, one (*P. ambigua*) is of uncertain taxonomic status (Kalininskaya & Golovacheva 1969) and the other is a laboratory genetic construct (Mergeay & Gerits 1978). Yet there seems to be no basic reason why diazotrophy should have bypassed all the pseudomonads despite the argument of Meganathan (1979) that cyanide formation might interfere with diazotrophy in some strains.

TABLE 1. A LIST OF BACTERIAL GENERA THAT INCLUDE DIAZOTROPHIC SPECIES

(Cyanobacteria or blue-green algae are listed as aerobic phototrophs; the heterotroph list incorporates some facultative chemolithotrophs.)

HETEROTROPHS	PHOTOTROPHS
Alcaligenes	(anaerobic)
Aquaspirillum	*Amoebobacter*
Arthrobacter	*Chlorobium*
Azomonas	*Chromatium*
Azospirillum	*Ectothiorhodospira*
Azotobacter	*Pelodictyon*
Azotococcus	*Rhodomicrobium*
Bacillus	*Rhodopseudomonas*
Beijerinckia	*Rhodospirillum*
Campylobacter	*Thiocapsa*
Citrobacter	*Thiocystis*
Clostridium	(aerobic)
Derxia	*Anabaena*
Desulfotomaculum	*Calothrix*
Desulfovibrio	*Chlorogloeopsis*
Enterobacter	*Chroococcidiopsis*
Erwinia	*Cylindrospermum*
Escherichia	*Dermocarpa*
Frankia	*Fischerella*
Klebsiella	*Gloeothece*
Methylobacter	*Lyngbya*
Methylococcus	*Myxosarcina*
Methylocystis	*Nostoc*
Methylomonas	*Oscillatoria*
Methylosinus	*Phormidium*
(*Pseudomonas*?)	*Plectonema*
Rhizobium	*Pleurocapsa*
Thiobacillus	*Pseudanabaena*
Xanthobacter	*Scytonema*
	Spirulina
	Synechococcus
	Xenococcus

Biochemistry of N_2 *fixation*

The primary enzyme responsible for diazotrophy is called nitrogenase. It has been isolated from 20–30 microbes as crude preparations and purified from about six. Its properties are much the same no matter what microbe it comes from, and the two proteins will usually (though not always) form an active enzyme even when obtained from different genera of microbe. The enzyme is binary, consisting of two brown metalloproteins which together reduce N_2 to $2NH_3$. The larger is an $\alpha_2\beta_2$ tetramer ($M_r \approx 2.2 \times 10^5$) containing both Mo (2 atoms/mol) and Fe (*ca.*

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32 atoms/mol) as well as sulphide (*ca.* 30 atoms/mol); the smaller is an α_2 tetramer ($M_r \approx 5 \times 10^4$) containing Fe (4 atoms/mol) and S (4 atoms/mol). Much is known of their physical biochemistry and understanding of their mode of action is well advanced, though incomplete. The probability is that N_2 is bound by the MoFe-protein, which may thus be the 'true' dinitrogenase†; the binding site is probably a metal atom, possibly Mo in a separable fragment of the MoFe-protein called 'FeMoco'. Both nitrogenase proteins are rapidly and irreversibly destroyed by exposure to O_2. Both proteins are essential for activity; small molecules such as acetylene, HCN and N_2O can replace N_2 as reducible substrates; if no reducible substrate is available, nitrogenase reduces the H_3O^+ ion and evolves N_2. Table 2 lists examples

TABLE 2. EXAMPLES OF SUBSTRATES REDUCED BY NITROGENASE

dinitrogen	($N_2 \rightarrow NH_3$)
acetylene	($C_2H_2 \rightarrow C_2H_4$)
hydrogen cyanide	($HCN \rightarrow CH_4 + NH_3 + CH_3NH_2$)
methyl isocyanide	($CH_3NC \rightarrow CH_4 + CH_3NH_2 + C_2$ products)
hydrogen azide	($HN_3 \rightarrow N_2 + NH_3 + N_2H_4$)
nitrous oxide	($N_2O \rightarrow N_2 + H_2O$)
cyclopropene	($\triangle \rightarrow \triangle + CH_3CH{=}CH_2$)
hydrogen ions	($H_3O^+ \rightarrow H_2$)

of reducible analogues of N_2. CO is a structural analogue of N_2 but is not reduced; it inhibits the reduction of all substrates but H_3O^+. Acetylene reduction has provided the now familiar 'acetylene test' for diazotrophy, which has revolutionized understanding of the subject. The H_2 evolution reaction persists to some extent even if N_2 is available; if D_2 is present, an exchange leading to HD formation accompanies N_2 reduction. In the cell, electrons are donated to nitrogenase by specific electron transport proteins (ferredoxins or flavodoxins), and biological energy (as ATP) is consumed when the enzyme functions, whether the substrate is N_2 or any of the alternatives listed in table 2. Finally, organisms do not synthesize nitrogenase if adequate NH_3 is available: NH_3 is a regulator of nitrogenase synthesis.

Physiology of N_2 *fixation*

The need for Fe and Mo, the requirement for ferredoxin or flavodoxin, the expenditure of ATP and, above all, the sensitivity of the proteins to damage by O_2, all impose physiological constraints on the N_2 fixation process. For example, N_2-fixing systems have unusually high requirements for Mo. The evolution of H_2 that accompanies N_2 fixation represents a 'waste' of diazotrophic potential because ATP is lost thereby; the more efficient microbes recapture the H_2 and use it as a supplementary substrate. Because of the ATP requirement of nitrogenase, more substrate (energy) must be diverted from general cell metabolism by a diazotroph in order to fix N_2 than it would require to assimilate preformed NH_3. The energy requirement for the nitrate reduction process is, however, not much different from that for N_2 fixation: see discussion

† Professor R. H. Burris and his colleagues (see Hageman & Burris 1980), accepting that the MoFe-protein binds N_2, have named this 'dinitrogenase' (after the correct chemical name for N_2, dinitrogen) and have named the Fe-protein 'dinitrogenase reductase'. A system comprising both proteins they still call 'nitrogenase'. This nomenclature is not yet accepted by all authorities (see Gibson & Newton 1981, p. 55) because a possibility still remains that the true 'dinitrogenase' is a complex of the two proteins.

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to Postgate & Cannon (1981, pp. 598–599). The overriding physiological constraint is the need to protect nitrogenase from damage by oxygen. Thus, several aerobic species of bacteria behave as microaerophiles or anaerobes when fixing N_2. A recent development is the discovery that many (usually slow-growing) rhizobia, once thought to be obligate symbionts when fixing N_2, are in fact only highly sensitive microaerophiles in these conditions and can fix *ex planta* at very low O_2 tensions. Well adapted nitrogen-fixing aerobes such as azotobacters use both respiration and reaction with a protective protein ('conformational protection') to screen nitrogenase from O_2; others use compartmentation, as in the heterocysts of cyanobacteria or the microaerobic nodules of legumes. In many organisms O_2 regulates nitrogenase biosynthesis, thus avoiding futile enzyme production in conditions physiologically unsuitable for nitrogen fixation. The oxygen relations of diazotrophs were reviewed by Robson & Postgate (1980).

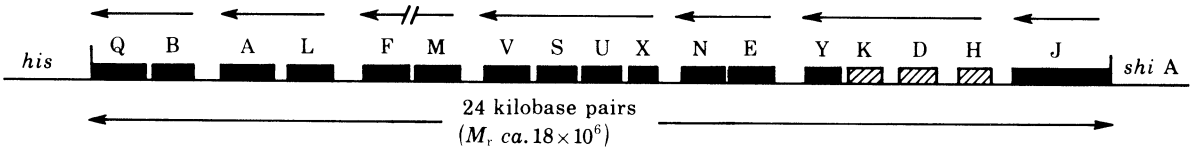

FIGURE 1. A map of the *nif* gene cluster of the *Klebsiella pneumoniae* chromosome. The letters signify the component genes (cistrons); the adjacent genes are *his* (for biosynthesis of histidine) and *shi*A (for uptake of shikimic acid). The arrows define the separate operons and the directions of transcription. The status of F and M as one or two operons is not wholly clear; two other genes, R and W, may be present (see text). The products of many have been 'seen' as spots on gel electrophoretograms of appropriate mutants (see table 3). The sizes of most of the genes are known (see Kennedy *et al.* (1981) for more details).

Genetics of N_2 *fixation*

K. pneumoniae has proved the most suitable organism for the study of the N_2 fixation genes, termed *nif* genes. The subject advanced explosively in the 1970s and is still developing. Figure 1 illustrates the map of *nif* in *K. pneumoniae*, strain M5a1, as of early 1981. The *nif* genes form a cluster located between *his* and *shi*A on the *K. pneumoniae* chromosome, which consists of 17 genes in seven or eight transcriptional units (or operons) at least one of which (J) is monocistronic. Brill (1980) includes two other genes in the *nif* cluster: *nif*R between L and F, and *nif*W between F and M. Table 3 lists the gene products and functions as far as they are known; the HDKY operon specifies the polypeptides of nitrogenase itself, and A and L are regulatory genes. Kennedy *et al.* (1981) reviewed the map of *nif* and its products.

Numerous mutations have been obtained in all the *nif* genes, including point mutations, deletions, polar mutations and insertion mutations. Recently an entirely foreign gene (*lac*Z) has been fused into each of the *nif* operons, an achievement that has greatly facilitated the study of their regulation (Dixon *et al.* 1980; Zhu & Brill 1980).

The techniques of handling recombinant DNA *in vitro*, sometimes called 'genetic engineering', have been used to isolate *nif* DNA and obtain a physical map; figure 1 combines both physical and genetic maps, which are entirely compatible. The whole *nif* cluster, and fragments thereof, have been 'spliced' onto small multicopy plasmids, so relatively large amounts of concentrated *nif* DNA can readily be prepared, radioactively labelled if required. The study of the products of cloned *nif* led to the discovery of the genes *nif*X and *nif*Y (Pühler *et al.* 1979; Pühler & Klipp 1981), whose functions are still obscure.

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The *Klebsiella nif* cluster has been transferred genetically to self-transmissible plasmids. These have been essential to all the mapping, regulation and recombinant DNA studies mentioned so far. They have also been used to generate completely new species of N_2-fixing bacteria – indeed, the *nif* plasmids were all constructed in *E. coli*, which is not known to fix N_2 in the natural environment. New 'hosts' for *K. pneumoniae nif*, which then fixed N_2, include *Salmonella typhimurium*, *Serratia marcescens*, *Erwinia herbicola*, a putative *Pseudomonas fluorescens* and *nif*$^-$ mutants of *Azotobacter vinelandii*; hosts that did not fix N_2 include *Proteus mirabilis* and *Agrobacterium tumefaciens* (see Postgate 1981).

TABLE 3. *NIF* GENE PRODUCTS IN *K. PNEUMONIAE*

(See also Kennedy *et al.* (1981).)

gene	$10^{-3} \times M_r$ of product	function or other comments
Q	?	not known
B	?	involved in synthesis or processing of FeMoco
A	57	regulatory: *nif*A product activates other operons
L	45	regulatory: some *nif*L 'revertants' escape O_2 regulation
F	17	a flavoprotein involved in electron input into nitrogenase
M	28	involved in processing of Fe protein
V	42	influences specificity of MoFe-protein
S	45	not known
U	32	not known
X	18	not defined genetically: presence deduced from physical map and products of cloned *nif* DNA
N	50	like B
E	46	like B
Y	19	discovered in the same way as X
K	60	specifies β subunit of MoFe-protein
D	56	specifies α subunit of MoFe-protein
H	35	specifies subunit of Fe protein
J	120	involved in electron input into nitrogenase?

The regulation of the *nif* cluster is complicated. Until recently, NH_3 was thought to act by influencing the composition of glutamine synthetase, which is an enzyme (synthesizing glutamine) and was considered also to be a regulator of *nif* and of some other genetic systems involved in the metabolism of N sources by microbes. In the presence of NH_3, glutamine synthetase reacts with ATP and loses its enzymic and, it was thought, its regulatory functions. Glutamine synthetase is specified by a gene (*gln*A) remote from *nif*: very recently, evidence has appeared that two other genes, *gln*L and *gln*G, are linked with *gln*A and it is probable that combinations of *gln* gene products, not glutamine synthetase, are the regulators.† Oxygen also regulates *nif* expression (*K. pneumoniae* does not make nitrogenase in the presence of dissolved O_2) by a mechanism that apparently bypasses products of the *gln* cluster. Once the *nif* cluster is 'switched on' by *gln* product or products, the operons within the *nif* cluster are regulated

† New views on the regulation of genes concerned in nitrogen assimilation are emerging at the time of writing and were expounded at session 70 of the 81st Annual Meeting of the American Society for Microbiology, 1–6 March 1981.

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by more subtle processes involving products of *nif*A (an activator?), *nif*L (a repressor?) and probably other genes (e.g. autoregulation occurs within the YKDH operon).

Expression of *nif* is thus regulated both internally (by *nif*A and *nif*L, among others) and externally (by *gln* genes). Among other genes that are outside *Klebsiella nif* but whose products are necessary for *nif* expression are *nar*D (involved in Mo processing), genes such as *unc* and one in *his*, which influence ATP supply, and a gene of uncertain function near *trp*, termed *nim* (Close & Shanmugam 1980).

Although most is known about *nif* in *Klebsiella*, information is now accumulating about *nif* genes in other microbes. Nif$^-$ mutants have been obtained in several genera of N_2-fixing bacteria including *Azotobacter vinelandii*, *Azospirillum lipoferum*, *Clostridium pasteurianum*, *Rhodopseudomonas capsulata*, *Rhizobium japonicum*, *R. leguminosarum* and some cyanobacteria. In *A. vinelandii* the *nif* genes may not be clustered (Bishop & Brill, 1977). The absence of established quantitative gene transfer systems at one time slowed progress with such organisms but the discovery of transformational gene transfer in *Azotobacter*, the 'gene transfer agent' of *Rhodopseudomonas* and promiscuous plasmids capable of mobilizing chromosomal material in rhizobia and *Azospirillum* accelerated progress in the late 1970s. Most recently the availability of cloned *Klebsiella nif* DNA has allowed very rapid progress, avoiding much laborious conventional genetics. The biochemical uniformity of the nitrogenase proteins reflects the fact that parts of the DNA specifying them are homologous, and this homology can be exploited to locate *nif* genes in new organisms. Thus the structural genes for *nif* in *Rhizobium leguminosarum* and other rhizobia have been shown to occur naturally on large plasmids (Nuti *et al.* 1979; Dénarié *et al.* 1981); in the cyanobacterium *Anabaena* 7120 they are chromosomal but apparently in a different order than in figure 1 (Mazur *et al.* 1980).

Homology with *Klebsiella nif* DNA can be exploited to 'clone out' *nif* genes from other organisms, and it is probable that recombinant DNA technology exploiting cloned *K. pneumoniae nif* will allow rapid localization and mapping of *nif* in many microbes in the next few years. Sequencing of cloned *nif* DNA has already provided the sequence of the Fe protein of *K. pneumoniae*, without the need to sequence the peptide itself (Sundaresan & Ausubel 1981).

Ancillary processes

Symbiosis and effectiveness

Environmentally the most important N_2-fixing systems are symbiotic and photosynthetic. The physiology of symbiotic systems, the modes and routes of infection and the bases of specificity are complex matters, which are dealt with in the symposia cited at the beginning of this article and surveyed in Sprent's (1979) book. Developments have been particularly rapid in the legume symbiosis, where genetics has provided useful insights. The genetics of symbiosis in rhizobia required first the conventional mapping of the rhizobial chromosome; in *R. leguminosarum*, *R. phaseoli* and *R. trifolii* the maps prove to be extremely similar, with *R. meliloti* not very different. At the end of the 1970s the discovery of large plasmids in rhizobia assumed great importance with the realization that not only *nif* (above) but genes determining host specificity may be plasmid-borne. In a strain of *R. leguminosarum*, a plasmid carrying bacteriocin genes (*cin*) also confers infectiveness for peas (*inf*), and genetic transfer of this plasmid to *R. trifolii* will change the host's specificity from clover and cause it to form ineffective nodules on peas. The correlation between *cin* and *inf* plasmids is not, however, universal. Much of the newer information on plasmids and diazotrophy in rhizobia is available in Gibson & Newton (1981).

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Some classes of nodules are ineffective through lack of leghaemoglobin; this protein is specified by the plant genome, though infection by the symbiotic bacteria is necessary to initiate synthesis of the apoprotein and the bacteria probably contribute the prosthetic group. Many mutations in rhizobia are known that appear to influence symbiotic effectiveness non-specifically; only one is known which has been characterized biochemically as *nif*$^-$. Mutations in the host-recognition system of *R. japonicum* have been obtained (Maier & Brill 1976). The genetics of rhizobia, both fast-growing and slow-growing, is now as active a research area as the genetics of diazotrophic *K. pneumoniae*.

Uptake hydrogenase

I mentioned earlier the fact that nitrogenase evolves H_2, which leads to inefficiency since the side-reaction consumes ATP and is wasteful. The most efficient rhizobia, like azotobacters (Yates *et al.* 1980), possess an uptake hydrogenase (i.e. a hydrogenase that takes up, but does not evolve, H_2) and re-use this H_2 to generate more ATP and generally to augment the efficiency of N_2 fixation (Dixon 1978; Evans *et al.* 1980). Mutants defective in the uptake hydrogenase genes (*hup*) of *Rhizobium japonicum* have been obtained that show lowered yields in glasshouse and pot tests (Evans *et al.* 1980); many natural strains of rhizobia lack *hup* genes or express them only weakly (Ruiz-Argüeso *et al.* 1978). The *hup* genes of *R. leguminosarum*, when present, are on the *inf cin* plasmid (Brewin *et al.* 1980). An unexpected consequence of the possession of *hup* genes is that *hup*$^+$ strains of *R. japonicum* can grow autotrophically, exploiting the H_2–O_2 reaction. *Derxia* species (an oxygen-sensitive genus of the aerobic Azotobacteraceae) is a similar facultative autotroph, but other *hup*$^+$ genera (*Beijerinckia*, *Azotobacter*, *Azospirillum*) are not (see Yates *et al.* 1981). The involvement of hydrogen in diazotrophy was reviewed by Robson & Postgate (1980).

Oxygen exclusion

The physiology of oxygen exclusion was mentioned earlier. O_2 is a regulator of *nif* expression not only in *K. pneumoniae* but also in the aerobe *Azotobacter*. However, synthesis of the protective protein of *A. chroococcum* is not regulated alongside synthesis of the nitrogenase proteins. In heterocystous cyanobacteria, heterocyst formation as well as nitrogenase synthesis is regulated by NH_3, and many *nif*$^-$ mutants are defective in heterocyst formation (Het$^-$). O_2-sensitive mutant cyanobacteria have been reported and some have relatively O_2-permeable heterocyst membranes (Haury & Wolk 1978).

Molybdenum assimilation

Mo transport and storage proteins are present in *C. pasteurianum* (Elliott & Mortenson 1977) and doubtless in other diazotrophs. Mo-processing may be shared with other systems requiring Mo: in *K. pneumoniae*, the product of *nar*D (incorporating Mo for nitrate reductase) is probably also involved in nitrogenase synthesis. Molybdate is not itself a regulator, but a molybdoprotein is probably involved in the regulation of at least one of the *Klebsiella nif* operons (*nif*YKDH). These matters were discussed in the review by Postgate *et al.* (1981). The molybdenum atom finally appears in the 'FeMoco' fragment of the MoFe protein and the products of genes *nif*B, N and E are among those involved in processing FeMoco.

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Relevance to the nitrogen cycle

Biological N_2 fixation is a fundamental step in the biological nitrogen cycle and is a rapidly advancing area of basic research. Developments in this area have already radically altered ideas about the ecology of N_2 fixation: whole new groups of diazotrophs, free-living and symbiotic, have been discovered and, despite certain disadvantages, the acetylene test has rendered N_2 fixation quantitatively the best understood step of the nitrogen cycle. I have illustrated these developments recently (Postgate 1980*a*, *b*) and shall not repeat myself here. Two considerations relevant to the future of the nitrogen cycle may be of interest to the present discussion.

Table 4. Distribution of diazotrophy in selected prokaryotic genera

genus (species)	number surveyed	number diazotrophic	percentage diazotrophs
Klebsiella (*aerogenes*, *pneumoniae*, etc.)‡	134	38	28
Erwinia (*herbicola*)§	18	4	22
Bacillus (*polymyxa*, *macerans*)‡	35	29	82
Desulfovibrio (five species)‖	10	7	70
Pleurocapsa†	12	7	58
Plectonema† group	25	16	64
Gloeothece†	5	5	100

† Non-heterocystous cyanobacteria; from Rippka & Waterbury (1977).
‡ See Postgate (1981).
§ Papen & Werner (1979).
‖ See Postgate (1974).

Distribution of N_2*-fixing ability*

The ease with which *nif* can be transferred between species and genera of microbes in the laboratory implies that such transfer also occurs in the natural environment. About 30% of 'natural' isolates of *Klebsiella pneumoniae* fix N_2, as do about 20% of *Erwinia herbicola*, so in these examples the natural gene pool of *nif* is smaller than the gene pool of the host species. Table 4 illustrates this situation for an arbitrary selection of prokaryotic genera for which diazotrophy is not a taxonomic character. The haphazard distribution of diazotrophy within prokaryotic genera and species indicates that diazotrophy is a property that is lost and gained fairly readily in Nature, at least among prokaryotic species. It is also likely that it is exchanged in Nature, even between genera. This view would account for the biochemical similarities of nitrogenase proteins from diverse sources: they are more closely related than highly conserved proteins such as eukaryotic cytochromes and haemoglobins (Eady 1977) and the four Fe proteins that have now been sequenced show high degrees of sequence homology (Scott *et al.* 1981). The *nif* cluster and parts thereof have been transferred in the laboratory by all the well known methods of microbial genetics (conjugation, transformation and transduction). The identification of the nitrogenase genes (equivalent to *nif*KDH) on natural plasmids in rhizobia emphasizes the likelihood of comparable *nif* gene transfer in the natural environment. However, a plasmid location is obviously not a prerequisite for transferability. Recent developments in *nif* genetics converge to suggest that the transfer of *nif* between chromosome and plasmid, performed artificially in the laboratory, can occur in Nature. The *nif* cluster or some operons therein, or both, seems to have features in common with a transposon. Evidence for 'inverted repeats' bounding *Klebsiella nif* tends to confirm this view (Pühler *et al.* 1979).

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The recognition of other determinants (*inf*, *hup*) on rhizobial plasmids suggests that properties such as host specificity may be interchangeable in symbiotic systems. All fast-growing rhizobia so far examined have plasmids; what species would a plasmid-free *R. leguminosarum* resemble, if any? Would it be distinguishable from a plasmid-free *Agrobacterium*? To generalize: a microbiologist would have difficulty in recognizing, say, a *nif*-deleted *Azotobacter* or *Clostridium pasteurianum* in Nature, so even such 'well defined' species may be only *nif*$^+$ representatives of a larger generic or specific microbial gene pool.

The conclusion that N_2 fixation and ancillary genes are mobile in Nature is strongly indicated, with the corollary that *nif*$^+$ and symbiotic bacteria will appear, sometimes as completely new genera, where selection is favourable.

However, some genera of microbes seem not to express *Klebsiella nif* (e.g. *Proteus*, *Agrobacterium*). Further, the prokaryotic–eukaryotic barrier seems not (yet) to have been crossed. Although a few authentic examples now exist of single genes from a eukaryote being expressed in a prokaryote, and of the reverse, regulated expression of a prokaryotic polycistronic multi-operon such as *Klebsiella nif* in even the simplest eukaryotic genome seems a highly improbable event in the present state of knowledge. Consistent with this thought, Elmerich *et al.* (1981) introduced *Klebsiella nif* on a recombinant cosmid into *Saccharomyces cerevisiae*, where it was conserved for 50 generations without expression. Modified and simplified *nif* systems capable of expression in eukaryotes are, of course, conceivable; constructs of this kind are the subject of some current research.

Future of agriculture

The need for a massive increase in N input into the World's agricultural soils over the next few decades, to parallel the rising World population, and the economic and environmental problems that this will entrain, have been discussed exhaustively (see Postgate 1980*c* and references therein). Though use of N fertilizer will increase, there is a high probability of a global swing towards more intensive exploitation of biological N_2 fixation. Modern knowledge of the fundamentals of diazotrophy is already facilitating such exploitation, a process that may be expected to continue; these matters were discussed recently at the Royal Society (Postgate & Cannon 1981). For example, genetically screened (e.g. *hup*$^+$) and later genetically improved inocula for established legumes are likely to become available in future, as are organisms genetically altered so as to be able to form agronomically significant biocoenoses with non-legumes such as cereals. Plant genetics will be quite as important as microbial genetics in determining the rate of innovation in such contexts, though existing systems could probably be exploited more than they are today. Somatic hybridization may in time yield new species of plant capable of symbiotic N_2 fixation; if the *nif* cluster can be rendered expressible in any eukaryote then the possibility of constructing crop plants capable of regulated *nif* expression, and thus independent of bacteria, will become considerably more real.

Nitrogen-fixing systems showing unregulated expression (e.g. *nif*-constitutive phototrophic bacteria) may have local value, perhaps in biotechnological installations, but they are not likely to be widely applicable because they are biologically uneconomic. Applications of fundamental knowledge are thus likely to contribute to an alteration of the character of N inputs and to changes in the environmental consequences thereof; some easing of the latter may be expected but no dramatic shifts, nor catastrophes. The total inputs will still be determined primarily by food requirements.

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