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Ecology and Biotechnology of Microbial Nitrogen Fixation**

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Seventy-eight percent of the air we breathe in is molecular nitrogen (N_2) – and the same amount we breathe out. Thus, human beings do not have the privilege of fixing N_2. Rather, this is the domain of the diazotrophic bacteria. Without these bacteria life on earth would not be possible. On a global basis they replenish the nitrogen content in soil by about the same amount that is lost by the process called denitrification. This article describes some of the biochemical and genetic complexity of the nitrogen fixation process, and summarizes a few more recent data on the molecular basis of nodule induction on legumes by the rhizobia. Finally, a critical evaluation is presented on the perspective to utilize research on biological nitrogen fixation for practical application.

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1. A Tribute to an Analytical Method

As this paper is presented on the occasion of a Meeting on Analytical and Applied Chemistry its first section is devoted to an analytical method whose discovery marks a historic milestone in biological nitrogen fixation research: the acetylene reduction assay. It was published in 1968 by *Hardy* et al.[1] and makes use of the unique property of the nitrogen-fixing enzyme, the nitrogenase complex, to reduce not only molecular nitrogen (N_2) to ammonia (NH_3) but also acetylene (C_2H_2) to ethylene (C_2H_4). The latter two compounds are easily separated and detected by gas chromatography thus making this assay one of the simplest, fastest, and most inexpensive enzyme tests known today. Furthermore, it can be applied *in vivo* with whole cells or even whole plants owing to the apparently free diffusion of substrate and product through barriers such as cell walls and membranes.

For qualitative determinations the acetylene reduction assay is certainly unbeatable; to measure nitrogen fixation quantitatively it has a few drawbacks that shall not be discussed in depth here. Without this method the enormous progress in biological nitrogen fixation research over the last 15 years would not have been possible, and since its discovery the acetylene reduction assay has appeared in the methodology section of literally thousands of publications.

After these somewhat unusual introductory comments the contents of the following sections will be almost entirely of biological rather than chemical nature, and will stick to the title of this article.

2. The Biological Nitrogen Cycle

There are numerous ways to illustrate the nitrogen cycle in nature. The one preferred by the author is shown in Fig. 1. In this scheme the inorganic nitrogen compounds are arranged from top to bottom by their oxidation states. The individual interconversions – most of them performed exclusively by microbes – are either oxidations or reductions, in which reduced or intermediate forms ($NH_4^{\oplus}$ and $NO_2^{\ominus}$) can be used as electron donors for aerobic energy production whereas the oxidized forms ($NO_3^{\ominus}$, again $NO_2^{\ominus}$, and N_2O) can serve as electron acceptors in energy production by anaerobic respiration.

However, not all reactions shown are energy-yielding. In fact the conversion from N_2 to NH_3, called nitrogen fixation, is one of the most energy-consumptive reactions in biology (see also Section 5).

3. The Ecological Role of Biological Nitrogen Fixation in the Nitrogen Cycle: Replenishing the Nitrogen Content in Soil

In undisturbed ecosystems and in old forms of agriculture which are based on natural processes, the nitrogen cycle as depicted in Fig. 1 is well sufficient to maintain the necessary quantities of nitrogen in the soil. In such natural cycles the fixation of atmospheric nitrogen plays an absolutely indispensable role in the replenishment of nitrogenous compounds. As shown in Table 1, the process called denitrification, i.e. the anaerobic reduction of nitrate to gaseous molecules such as N_2O and N_2 (reaction 6 in Fig. 1) accounts for

Table 1. Quantities of nitrogen turned over annually on earth (from Ref.[2,3]).

Process	tons per year
Biological nitrogen fixation	2×10^8
Chemical nitrogen fixation	6×10^7
Atmospheric nitrogen fixation	1×10^7
Denitrification	1 to 2×10^8

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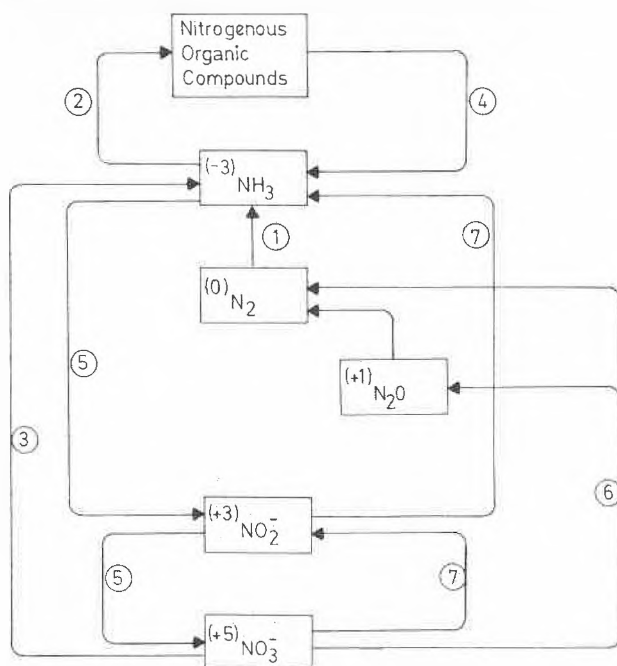

Fig. 1. Biological interconversions in nature's nitrogen cycle. The inorganic nitrogen compounds are arranged (from top to bottom) by their oxidation states. (1) Nitrogen fixation; (2) ammonium assimilation; (3) assimilatory nitrate reduction; (4) ammonification (by bio-degradation); (5) nitrification; (6) denitrification; (7) nitrate/nitrite-ammonification. Modified from Ref. [19].

an estimated 1 to 2 × 10^8 tons on earth of nitrogen lost annually to the atmosphere. Owing to the process of nitrogen fixation approximately the same amount of nitrogen, or even a bit more, is brought back to the soil via N_2-fixing (diazotrophic) microorganisms[2,3].

The most radical disturbance of the natural nitrogen cycle today is the man-made world agricultural production which would rapidly lead to nitrogen limitation if it were not subsidized by a significant input of nitrogenous fertilizers (Table 1) produced in industry by fixing nitrogen chemically. Another source of nitrogen input to the soil (ca. 1 × 10^7 tons per year) is the precipitation of nitrogen oxides from the atmosphere generated by lightning, natural combustion (e.g. forest fires), and anthropogenic pollution.

From ancient times biological nitrogen fixation has played a predominant role in agricultural production by the empirical use of legumes (which fix N_2 symbiotically) as green manure. In countries practicing modern agriculture this technique has largely been replaced by the use of chemical fertilizers. Only recently a renewed interest in biological nitrogen fixation is developing because it has been recognized (i) that fossil fuels as energy source for chemical N_2 fixation should not be wasted for a process that can be driven by solar energy (*viz.* symbiotic N_2 fixation by plants), and (ii) that chemical nitrogen fertilizers, if not carefully applied, contribute to pollution by leaching into natural waters.

Although no one seriously expects that nitrogen fertilizers will be entirely replaced by biological N_2 fixation, it is nevertheless believed that modern microbiological research on N_2-fixing bacteria could help to make more extensive and more efficient use of existing diazotrophic systems for the benefit of agriculture. Applicable results from such research should, in particular, be made available to poor countries in which the demand for increased food production is higher than anywhere else.

4. N_2 Fixation is the Monopoly of Prokaryotes

Within the three kingdoms of living organisms, the eukaryotes, the eubacterial and the archaebacterial prokaryotes, N_2 fixation is carried out only by members of the two prokaryotic kingdoms. Table 2 shows a selection of diazotrophic bacteria[3,4]. An important, recent addition to this list was the detection of nitrogen fixation in methanogenic archaebacteria[5,6].

Table 2 shows that the ability to fix molecular nitrogen is distributed over a wide range of phylogenetically distant and physiologically diverse bacteria. Although the nitrogenase reaction in these bacteria is uniformely oxygen sensitive, it is remarkable that even aerobic bacteria have learned to cope with that problem by having developed various means of protecting nitrogenase against the detrimental action of oxygen. The free-living diazotrophs are able to use N_2 as the sole source of nitrogen which implies that they readily assimilate the product of the nitrogenase reaction, $NH_4^{\oplus}$. In their natural habitats (soil, water) these bacteria contribute only little to the global input of N_2 from the atmosphere. In contrast, on a global basis, the majority of the N_2 is fixed by symbiotic systems.

The most well-known and agriculturally important system is the root-nodule symbiosis formed between bacteria of the genera *Rhizobium* and *Bradyrhizobium* and the legumes[7]. However, the contribution of the actinorhizal symbiosis between *Frankia* species (actinomycetes) and woody non-legumes (e.g. alder tree) for the fertility of forest soils does not deserve to be underestimated (see Table 3). All these true symbioses have in common that the $NH_4^{\oplus}$ derived from N_2 fixation is not assimilated by the bacteria but is exported to the plant cells in which the nitrogen is then incorporated into organic matter. Another system, the associative symbiosis of cyanobacteria with the water fern *Azolla*, has attracted much attention because of its importance in the fertilization of rice paddies.

Table 2. A selection of nitrogen-fixing (diazotrophic) bacterial species.

A. Free-living, heterotrophic bacteria

1. aerobic
   - *Azotobacter vinelandii*
   - *Azotobacter chroococcum*
   - *Derxia gummosa*
2. microaerobic
   - *Azospirillum brasilense*
   - *Methylococcus capsulatus*
   - *Thiobacillus ferroxidans*
3. facultative anaerobic
   - *Klebsiella pneumoniae*
   - *Enterobacter agglomerans*
   - *Bacillus polymyxa*
4. anaerobic
   - *Clostridium pasteurianum*
   - *Desulfotomaculum ruminis*
   - *Methanococcus thermolithotrophicus*

B. Free-living, phototrophic bacteria

- *Rhodobacter capsulatus*
- *Rhodopseudomonas palustris*
- *Chromatium vinosum*
- *Chlorobium limicola*
- *Anabaena variabilis*
- *Nostoc* sp.

C. Symbiotic bacteria[a)]

- *Bradyrhizobium japonicum* (soybean)
- *Rhizobium meliloti* (lucerne)
- *Rhizobium leguminosarum biovar viciae* (pea)
- *Rhizobium leguminosarum biovar trifolii* (clover)
- *Rhizobium leguminosarum biovar phaseoli* (bean)
- *Frankia alni* (alder)

[a)] The symbiotic bacteria use a microaerobic mode of metabolism when fixing nitrogen.

Table 3. Reported rates of N_2 fixation by specific symbiotic systems (from Ref.[15]).

N_2-fixing system	Symbiotic bacterium	kg N_2 fixed × ha^{-1} × yr^{-1} (approximately)
Soybean	*B. japonicum*	90
Clovers	*R. trifolii*	150
Lucerne	*R. meliloti*	150
Rice paddy	*Anabaena* sp.	40
Alder	*Frankia* sp.	100

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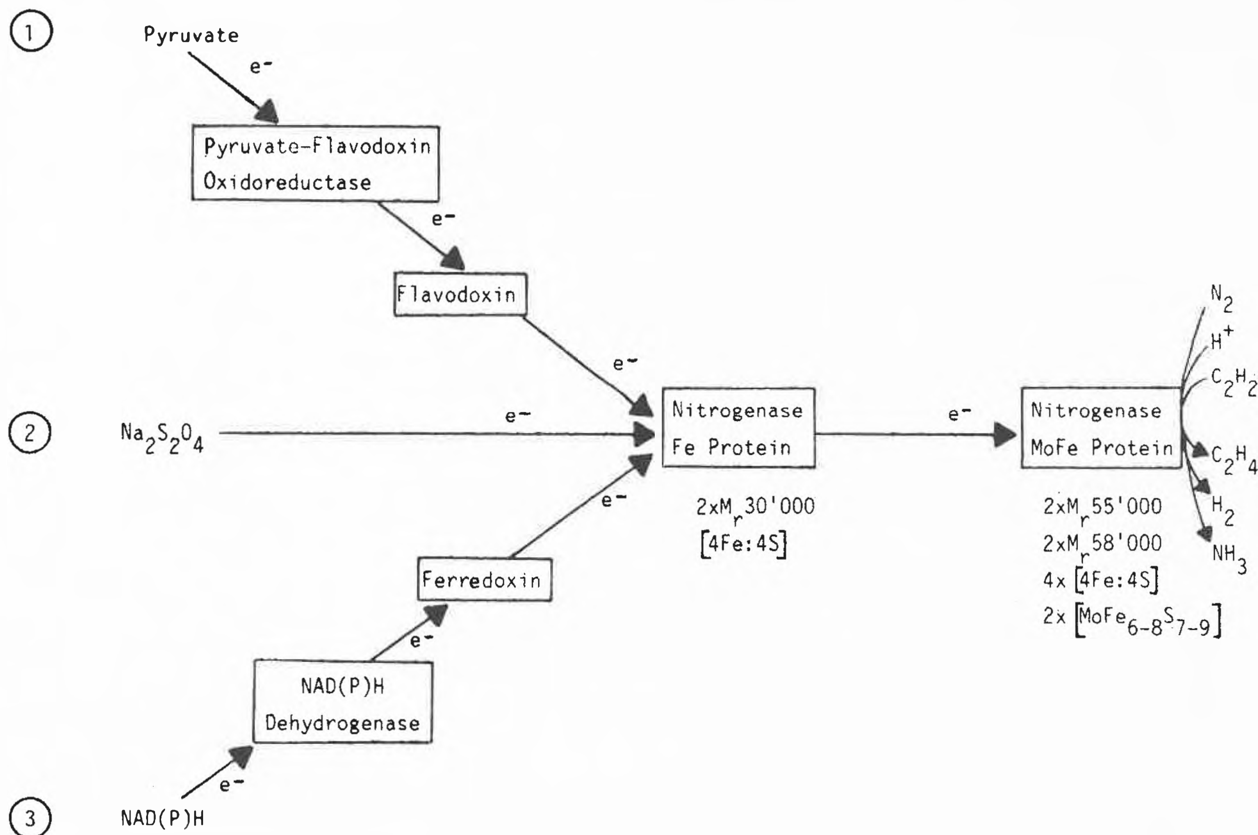


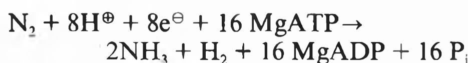
Fig. 2. Composition and function of nitrogenase, and routes of electron transport to nitrogenase. (1) In the facultative anaerobe, Klebsiella pneumoniae; (2) in vitro, i.e. in an anaerobic cell-free system with an artificial electron donor; (3) in aerobic diazotrophs, e.g. Rhizobium. The transfer of an electron from the Fe protein to the MoFe protein consumes two molecules of MgATP (see text).

5. The Biochemical Complexity of Nitrogen Fixation

Despite the phylogenetic heterogeneity of diazotrophic bacteria, the nitrogenase complexes in these bacteria are remarkably similar[8]. In all species examined, nitrogenase consists of two oxygen-sensitive redox proteins (Fig. 2), one which contains molybdenum and iron (MoFe protein) and the other which contains iron (Fe protein). These cofactors are bound to the proteins via acid-labile sulfur in the form of 4Fe:4S clusters, and as an iron-molybdenum cluster (FeMo cofactor) which has a composition of $MoFe_{6-8}S_{7-9}$. The structure of the latter complex has not yet been elucidated. Circumstantial evidence suggests that molybdenum forms part of the active site involved in reducing N_2 and other substrates among which is acetylene (see Section 1). The MoFe protein is a tetramer consisting of two α and two β polypeptides whereas the Fe protein is a dimer of two identical subunits (Fig. 2).

Under molybdenum limitation species of the genus *Azotobacter* synthesize an alternative nitrogenase complex which contains vanadium[9], and which characteristically reduces acetylene to ethane (C_2H_6) albeit poorly. It cannot be ruled out at present that other organisms besides *Azotobacter* contain a similar vanadium-containing nitrogenase, too.

When the two component proteins combine *in vivo* or *in vitro* and are supplied with an appropriate source of low-potential reductant and with MgATP, the complex catalyzes the following overall reaction:



$$N_2 + 8H^{\oplus} + 8e^{\ominus} + 16\,MgATP \rightarrow 2NH_3 + H_2 + 16\,MgADP + 16\,P_i$$

The equation shows that two molecules of ATP are consumed per electron transferred.

Where the electrons are derived from *in vivo* depends on the cellular physiology of the bacterium. Generally, under anaerobic growth conditions (e.g. in *Clostridium* species or *Klebsiella pneumoniae*) a fermentation intermediate such as pyruvate serves as the electron donor to first reduce either a ferredoxin or a flavodoxin; subsequently, this reduces the Fe protein, which in turn reduces the MoFe protein (Fig. 2). In aerobic and microaerobic diazotrophs (e.g. in *Azotobacter*, *Rhizobium*, and *Bradyrhizobium* species) NAD(P)H is the likely electron donor. The electrons are transferred to a ferredoxin or a flavodoxin via a putative membrane-associated dehydrogenase (Fig. 2). The problem with this

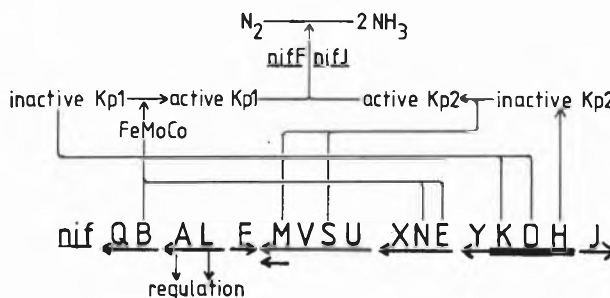


Fig. 3. The seventeen Klebsiella pneumoniae nitrogen fixation (nif) genes. The genes are located adjacent to each other (so-called «cluster») on the chromosome. Most genes are organized in multicistronic operons. The direction of transcription of the individual transcription units is shown by the arrows underneath the gene designations. The nitrogenase structural genes (nifH, nifD, nifK) are emphasized by a bold line. The functions of some of the genes are indicated. The products of nifJ and nifF are involved in electron transport, i.e. nifJ codes for the pyruvate-flavodoxin-oxidoreductase, and nifF for the flavodoxin (cf. Fig. 2).

idea is that the intracellular redox potential of NAD(P)H may not be low enough to reduce a protein like ferredoxin which also has a low mid-point potential. To overcome this problem the hypothesis has been put forward that an energized membrane helps to lower the potential of the electron donor[10].

6. The Genetic Complexity of Nitrogen Fixation

From the information given in the preceding section it becomes clear that, apart from the nitrogenase polypeptides, a substantial number of accessory proteins must be required for building up and supporting a catalytically active nitrogenase complex. It is thus not suprising that not less than 17 nitrogen fixation (*nif*) genes have been identified in the free-living diazotroph, *Klebsiella pneumoniae*[11]. In this organism the characterization of the *nif* genes has been facilitated because they are located closely together on a chromosomal DNA region of 23×10^3 nucleotides length (*nif* cluster; Fig. 3). The functions of many, but not all, *nif* genes is known (Fig. 3). Similar genes were identified in other nitrogen fixing bacteria in which they are organized in a different way as in *K. pneumoniae*[12].

The expression of the nitrogen fixation genes is tightly regulated by sophisticated control circuitries[11]. For example, from work done in the author's laboratory, the genetic control of N_2 fixation in the soybean root-nodule symbiont, *Bradyrhizobium japonicum*, is beginning to become understood (Fig. 4). The model in Fig. 4 involves a cascade of gene activation steps. In the first step a postulated master regulatory protein is supposed to activate the expression of an operon carrying the *nifA* gene. The *nifA* gene then determines the synthesis of another regulatory protein which activates all other *nif* genes in the second step. Interestingly, the second activation step is oxygen sensitive[13]. This way the cells ingeniously prevent the expression of most *nif* genes under a condition – high oxygen tension – unfavourable to the enzymatic reduction of N_2.

Why are all these details reported in the context of the title of this article? The idea is that a thorough knowledge about the enormous biochemical and genetic complexity of nitrogen fixation may help the reader to critically evaluate the feasibility or non-feasibility of any biotechnological endeavor aimed at an improvement of nitrogen fixation (see later in Section 9).

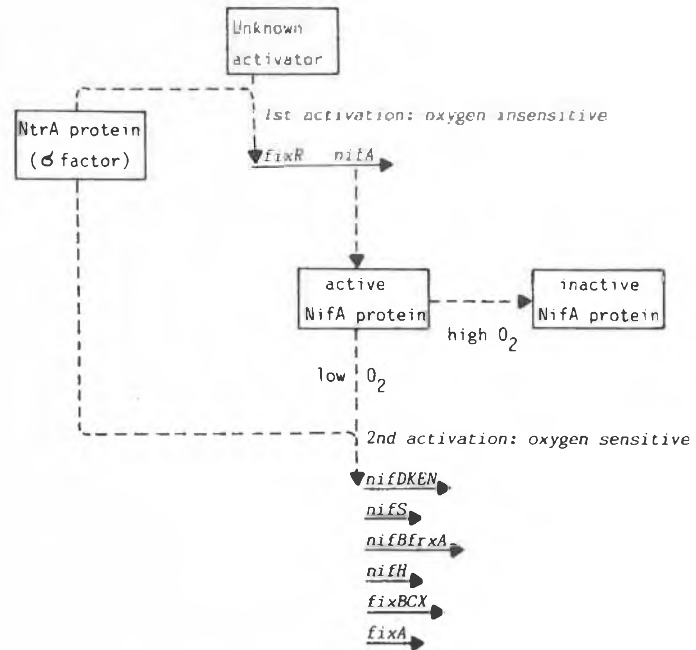


Fig. 4. Model for the regulation of nif and fix genes in Bradyrhizobium japonicum. Since all nif genes carry a characteristic nif consensus promoter, they all require a specific σ factor of RNA polymerase in order to become transcribed. In addition, the genes are positively controlled, i.e. they need to be activated by specific regulatory proteins. In a first activation step, the fixRnifA operon is activated by a protein that has not yet been identified. This step of gene expression is not sensitive to oxygen. After synthesis of the nifA gene product, the NifA protein serves as an activator of the transcription of a series of other nif/fix operons. This activation proceeds best under low oxygen tension, whereas at high oxygen concentration the NifA protein is converted to an inactive form, thus blocking transcription of the nif/fix operons.

7. How Does a Legume Nodule Form?

This is still one of the mysteries of nitrogen fixation research, even though there is a lot more known today due to the rapid progress in the genetics of the nodulation

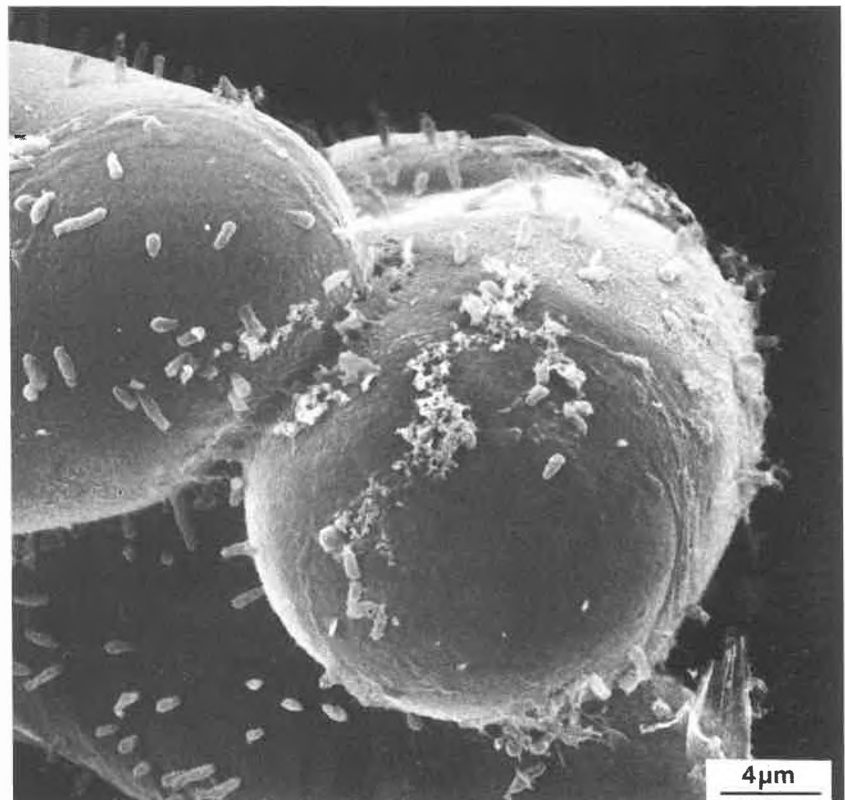


Fig. 5. A view on the surface of the tip of a soybean root hair by a scanning electron microscope. The attachment of rod-shaped bacteria (species Bradyrhizobium japonicum) is visible. The cleft in the root hair marks the beginning of the process called «root-hair curling» which is the first visible response of the plant to infection (Photograph: D. Studer).

process within the last 3 to 4 years[14]. The additional level of complexity is that there is a coordinated induction not only of bacterial but also of plant genes, and that the symbiotic systems differ by their host specificity which must be explainable in molecular terms.

In most root-nodule symbioses the different stages of nodule development follow similar routes. First, the bacteria (*Rhizobium* or *Bradyrhizobium*) attach to the surface of the root hair (Fig. 5). As a first visible plant response, this elicits the curling of root hairs. Then, an infection thread is formed through which the bacteria migrate, until they are released from it into the plant cytosol. Concomitant with these events there is an increased cortical cell division which provides the basis for the nodule structure. In a completely differentiated nodule the infected plant cells are fully occupied by endosymbiotic bacteria (specially termed «bacteroids») which find themselves in an ideal environment (microaerobiosis, nutrients plentiful) to carry out the N_2-fixation reaction (Fig. 6).

Genetic studies have recently brought light into the molecular events taking place in the early steps of nodule formation[14]. A group of so-called «common» nodulation (*nod*) genes have been found in all rhizobia and bradyrhizobia investigated. Mutations in any of the genes *nodA*, *nodB*, and *nodC* lead to a complete block in root-hair curling and nodule formation. This shows that the products of the *nodABC* genes have a biochemical function that produces the hair curling response, but of what nature this function is, remains to be determined. The *nodABC* genes are not normally expressed. They need to be induced. Recently, low molecular weight compounds present in root exudates of the legume host plants have been identified which function as inducers. In the case of the interaction between *Rhizobium* species and the temperent legumes, flavonoid derivatives have been shown to be the most potent inducers. In the interaction between *Bradyrhizobium japonicum* and soybean the isoflavone daidzein (4',7-dihydroxyisoflavone) was found to be the best inducer of *nod* genes[16,17]. In order for induction to occur an intact *nodD* gene must also be present the product of which is believed to function as a transcriptional activator for the expression of the other *nod* genes.

A model which summarizes the current thinking is presented in Fig. 7 using the *B. japonicum*-soybean interaction. Thus, at least some detail in the molecular crosstalk between the two symbiotic partners has been elucidated. The plant sends out substances (flavones, isoflavones) which trigger the induction of the common *nod* genes. Once expressed, their products – directly or indirectly – turn on the first visible plant response called root-hair curling. It can be envisaged that more signals will be exchanged in the following processes of infection thread formation, cortical cell division, etc.

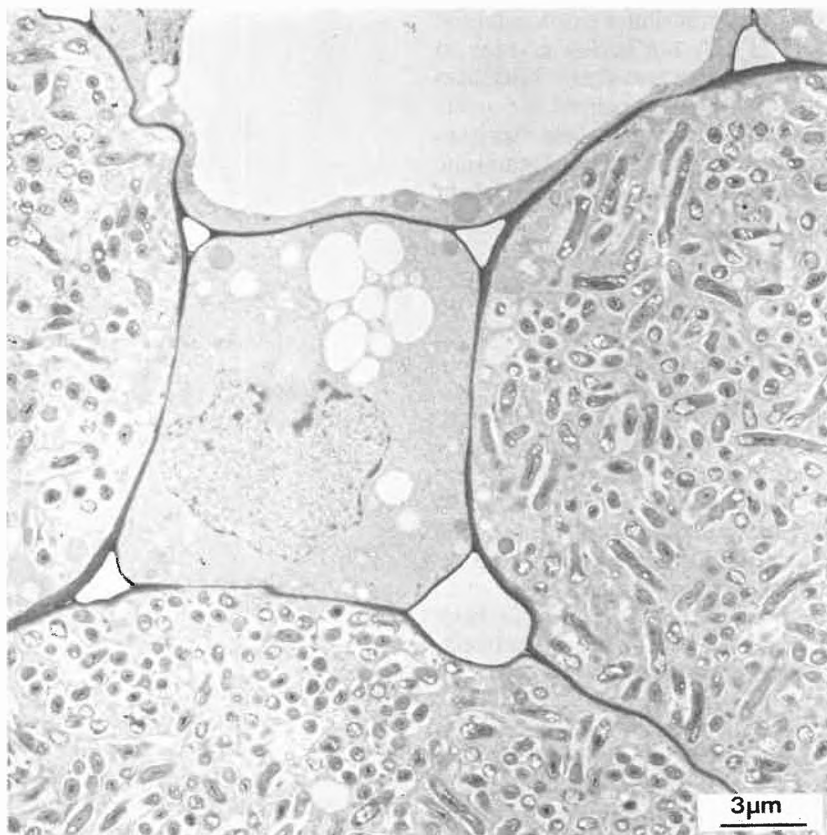


Fig. 6. A section from a soybean root nodule showing infected and uninfected nodule cells, viewed by transmission electron microscopy. In the infected plant cells the dense colonization by Bradyrhizobium japonicum cells is well visible (Photograph: D. Studer).

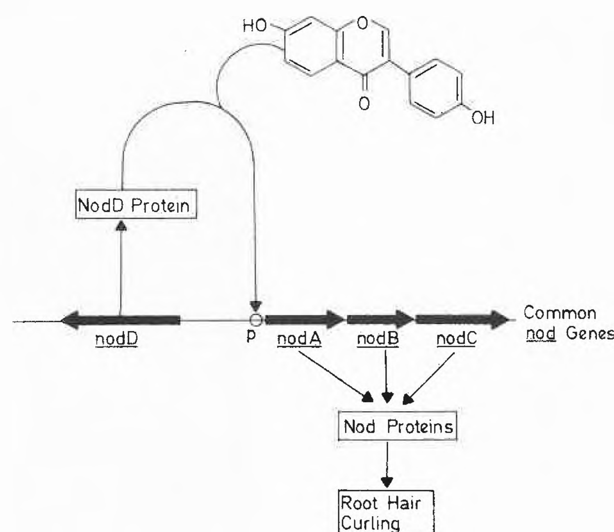


Fig. 7. Model for the induction of the nodulation genes in Bradyrhizobium japonicum. The phenolic compound shown on top is 4',7-dihydroxyisoflavone (daidzein) which is a component in the root exudate of soybean. Daidzein serves to induce the B. japonicum early nodulation genes (nodABC) with the help of the NodD protein. The nodD gene is divergently transcribed from the nodABC genes and presumably codes for a transcriptional regulator of the promoter (p) of the nodABC operon. It must be emphasized, however, that neither the binding of the isoflavone to the NodD protein nor the binding of the NodD protein to the nodABC promoter have been proven by in vitro experiments. The model is solely based on specific mutants with defects in nodD, and on gene expression experiments in vivo. After induction of nodABC, the corresponding gene products elicit the root-hair curling response.

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8. Nitrogen Fixation and Biotechnology Today

As indicated before the agriculturally most important crop plants which are able to derive their nitrogen supply from symbiotic nitrogen fixation are the legumes. Worldwide, grain legumes, for example, are grown on an area of 1.3 to 1.5×10^6 km^2 with a yearly production of about 200×10^6 tons[7]. Almost half of this amount is brought about by soybean.

For almost a century, inoculation of legumes by rhizobial cultures has been a common practice in agriculture to ensure adequate formation of the N_2 fixing root-nodule symbiosis. Today a number of seed companies have production facilities to grow specific *Rhizobium* and *Bradyrhizobium* strains with which the seeds are either directly coated, or a peat-based inoculum is prepared and sown together with the seeds. This market could assume quite significant dimensions if the inoculation practice was rigorously applied: for the inoculation of the approximately 0.6×10^6 km^2 of soybean plantations about 18 thousand tons of rhizobial cells would have to be produced!

There is, however, a problem with the inoculation practice: introduction of desired bacteria into the soil frequently results in failures mainly because of the presence of native bacteria which are more competitive for nodule-formation but often less effective in nitrogen fixation[18].

9. Genetic Engineering for Improved and Extended Nitrogen Fixation: Future Directions

The aforementioned dilemma brings into focus one of the most urgent problems to be solved by biotechnological research, i.e. to construct a suitable rhizobial or bradyrhizobial inoculum strain that performs best with the seed variety with which it is applied, and outcompetes native bacteria in a given type of soil and climate. Given the enormous varieties of different plant cultivars, soil conditions, and climatic regimes this will not be an easy task. Nevertheless, to pursue this goal is probably the only one which makes sense as all other endeavors such as transferring *nif* genes to new plants or establishing new symbioses are goals with an order of magnitude more complex to handle.

Which properties should an ideal rhizobial inoculum have?

(1) It should be a competitive nodulator. Perhaps it may become possible to identify key competition genes which, when constitutively expressed, give the strain a head-start in nodulation as compared to its competitors. Another way may be to utilize the knowledge about specific inducer molecules of nodulation, i.e. adding such inducer substances to the inoculum.

(2) It should be an efficient nitrogen fixer. Knowing the complex biochemistry and genetics underlying the nitrogen fixation process, it will not be easy to improve the nitrogen fixation rates of existing symbioses. There are two many-candidate biochemical steps which could be the rate-limiting ones and thus potential targets for improvement (electron transport, energy supply, trace element supply, etc.). Certainly, good knowledge about genetic regulation is a prerequisite before sensible genetic manipulations can be attempted.

Other desirable goals to reach, as discussed on and off in the past, rather must be classified into science fiction: the transfer of *nif* genes to create new N_2-fixing plants, or the transfer of the whole genetic information for the root-nodule symbiosis to other plants. The complexity of such an undertaking would be enormous. Although it may become possible to transfer, for example, all the *K. pneumoniae nif* genes to cereals and even get them expressed there, there are numerous other hurdles to take. Will there be an optimal supply of iron and molybdenum, of reductant and energy, and is the exclusion of oxygen guaranteed? Will the plant benefit from the implanted *nif* genes, or will it suffer from energy drainage to the costly nitrogenase reaction?

10. Conclusion

As in the past years nitrogen fixation will remain one of the most exciting areas of basic research in microbiology. There are numerous problems to solve such as the elucidation of the molecular mechanisms of symbiotic *nif* gene regulation, or the molecular mechanisms underlying the various steps in bacteria-plant interaction leading to the root-nodule symbiosis. It is, however, difficult to predict when this research comes to fruition with regard to practical application. The near-term goals are to improve the existing symbioses, for example, by creating superior rhizobia or bradyrhizobia for their use as inoculum.

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