ESSENTIALS OF NITROGEN FIXATION BIOTECHNOLOGY

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Summary

Nitrogen constitutes 78% of the Earth's atmosphere, yet it is frequently the limiting nutrient to agricultural productivity. This necessitates the addition of nitrogen to the soil either through industrial nitrogen fertilizers, which is accomplished at a substantial energy cost, or by transformation of atmospheric nitrogen into forms which plants can take up for protein synthesis. This latter form is known as biological nitrogen fixation and is accomplished by free-living and symbiotic microorganisms endowed with the enzyme nitrogenase. It is not only economically sound, but also environmentally more acceptable.

Biological Nitrogen Fixation (BNF) accounts for from 40 to 70% of the total global nitrogen input, yet many developing countries have not harnessed this potential fully. BNF has the potential to increase world food production through biofertilizers (see also BG 6.58.6 - *Environmental Biotechnology*), which is especially important in the developing countries where food shortages are common and prices of industrial nitrogen fertilizers are usually prohibitive. Research and development of BNF technology has been in existence for the last four decades in developing countries and has mostly been undertaken by specialized institutions like the regional Microbiological Resources Centers (MIRCENs). The BNF-MIRCENs are one of the UNESCO biotechnology programs, the main objective of which is to reinforce the conservation of microorganisms in developing countries with an agrarian base, with emphasis on *Rhizobium* gene pools; and to foster inexpensive technologies such as biofertilizers.
The identification, cloning and sequencing of the twenty nitrogen fixation (*nif*) genes directly involved in nitrogen fixation as well as the isolation of cell-free extracts of the nitrogenase enzyme has laid the foundation for a better understanding of the complex biochemical and physiological processes involved in the reduction of atmospheric nitrogen to ammonia using the enzyme nitrogenase. The development of techniques for handling the extremely oxygen-sensitive nitrogenase enzyme has resulted in detailed kinetic, physico-chemical and X-ray crystallographic analysis of the component nitrogenase proteins. This has paved way to the discovery of three genetically distinct, though related, nitrogenase systems; one based on molybdenum, another on vanadium, and a third one that functions without either Mo or V. The taxonomy of the rhizobia (initially based on host legume infection) is constantly being refined, and now encompasses phylogenetic analyses based on 16S rRNA gene sequencing which has now become the standard tool for classification of bacteria.

1. Introduction

Nitrogen fixation is a major life-supporting process on this planet. It is responsible for the conversion of "inert" dinitrogen (N_2) gas from the atmosphere into usable ammonia, replacing the fixed nitrogen constantly being lost to the atmosphere by the denitrification process. Worldwide agricultural productivity is determined by the availability of fixed N_2 in all its forms and upon which the continually increasing human population depends for its survival. There is no doubt that the population explosion in this century was supported primarily by the Haber–Bosch process for producing nitrogenous fertilizer, the Green Revolution in which high-yielding varieties of the major non-legumes of the world (wheat, rice and maize) were sold in the context of plentiful fixed nitrogen, and lastly on the improvement of health care the world over.

It has been estimated that such industrially fixed nitrogen supports at least a third of the human population at present. However, biological nitrogen fixation (BNF) provides perhaps two thirds of the nitrogen needs of the biosphere, much of it occurring naturally. Biological nitrogen fixation is the ability of living organisms to convert the inert dinitrogen gas of the atmosphere (N_2) into nitrogen-containing organic compounds, such as ammonia (NH_3) and nitrate (NO_3^-), that become available to all forms of life through the nitrogen cycle. Figure 1 shows a simplified version of the N-cycle.

It is now over 102 years since BNFwas shown to occur in legumes, and more than 40 years since the area of BNF became a significant area of international research. This followed the stimulus given when it became possible to study nitrogenase in cell-free preparations in the early 1960s. Later, the genes controlling the expression of nitrogen fixation became available to laboratory study and a new burst of research activity was initiated. Since the 1960s, improvements in agricultural technology have occurred, and perhaps the main practical impact of BNF has been in the provision of effective *Rhizobium* cultures for crop and pasture legumes, whereby the two form symbiotic associations resulting in the development of structures called *root nodules*, where the dinitrogen fixation process takes place (see, Figure 2). This has always been highly successful and, indeed, this area is by far the most significant contribution to agriculture involving biotechnology.

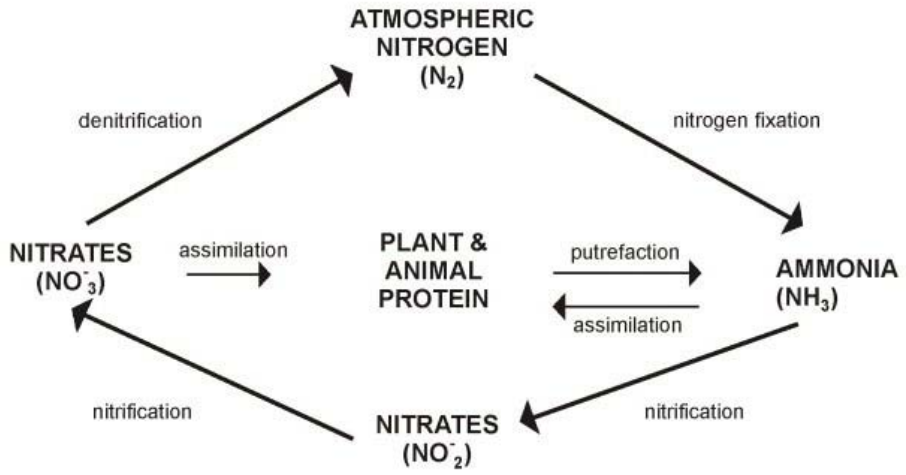

Figure 1. A simplified version of the nitrogen cycle

Figure 2. Legume root nodules on soybean.

In the same period (between 1950 and 1900), world fertilizer production increased tenfold, over half of the increase being nitrogen fertilizer applied to cereal crops. Such ready availability of fertilizer nitrogen (at a cost) may have encouraged a spirit of complacency in policy makers, detrimental to the promotion of BNF the resources made available in recent years by governments for BNF in developed countries seem to have been reduced. Despite the huge increase in nitrogen fixation in the past 35 years, most of this has been Industrial Nitrogen Fixation (INF) using fossil fuels to reduce atmospheric nitrogen to ammonia. In 1990, world consumption of fertilizer was 80 million tons. By 2020 (when Kenya hopes to be industrialized) it is anticipated that 160 million tons of nitrogen for cereal production will be required, despite a recent downturn in nitrogen fertilizer use during the early 1990s, mainly as a result of political changes.

2. Crop Requirements for Nitrogen

One ton of wheat grain removes approximately 20kg N from the soil. A good crop (5 tons of grain per ha^{-1}) removes 100kg N and an excellent crop (10tons grain ha^{-1}) removes 200kg N ha^{-1}. More than half the countries recorded currently grow cereals with yields of less than 3 tons of grain ha^{-1}, using fertilizer nitrogen application expressed as urea of 100kg ha^{-1} or less.

The soil crop nitrogen budget is very complex: some of the nitrogen in grain originates with soil nitrogen reserves, and some of the nitrogen in fertilizer goes to maintain or increase these reserves. Some nitrogen is lost to the environment (air and water) from

the soil crop system. Table 1 presents a nitrogen budget for growth of cereals in France, indicating the fate of nitrogen supplied as fertilizer.

Designation/category	**%**
Taken up by the crop	40 - 60
Incorporated in soil organic matter	20 - 50
Mineral forms in soil (clay - ammonium complex)	5 - 20
Lost by denitrification and volatilisation	2 - 30
Lost by leaching	2 - 10

Table 1. Fate of fertilizer nitrogen given to a crop.

These numbers will differ for other regions and crops (e.g. 30% or more of the nitrogen applied as urea to paddy rice can be lost to the atmosphere as ammonia). Highly productive agriculture requires close control of nitrogen supply to the crops to ensure that the yield potential is realised. Also, fertilizer costs are only a minor part of the total farming costs in developed countries. It is unlikely that BNF-based systems will be able to compete with mineral fertilizer as a plant nitrogen source for many years to come, for crops and in regions with highly productive agriculture. However, there are vast regions (e.g. much of sub-Saharan Africa) where yields are low and fertilizer use is minimal due to:-

- Erratic rainfall resulting in unpredictable crop yields.
- Fluctuating market prices for product, with low prices making bumper crops unprofitable.

Under such circumstances cash investments in farms (e.g. fertilizers) can be too risky for farmers. It is under these circumstances that BNF-based systems seem most promising and potentially profitable. However, it must be recognized that nitrogen is not the only limiting nutrient. Increasingly, phosphorus emerges as a yield limiting factor in Africa. Phosphorus is often the most limiting factor second only to water. To use BNF or give crops new BNF capacity is equivalent to unbalanced nitrogen fixation. This is well known to be detrimental to soil fertility.

3. Potential for Biological Nitrogen Fixation [BNF] Systems

Although a diverse range of nitrogen-fixing systems has now been recognized, BNF is restricted to prokaryotes and has never been found in eukaryotic organisms (Table 2). Among prokaryotic organisms, some achieve nitrogen fixation on their own, whereas others must establish a symbiotic relationship with a eukaryotic system to support fixation. The range of separate microbes that can fix nitrogen is now recognized to involve hundreds of species, covering most of the different biotrophic energy systems from photosynthetic energy bacteria (e.g. *Rhodospirillum rubrum*), anaerobic bacteria (C*lostridium spp.*, *Beijerinckia spp.*), microaerophilic *(Azospirillum*, *Herbaspirillum*, *Acetobacter*, *Azorhizobium*, *Azoarcus*, *Burkholderia*, etc), and aerobic bacteria (*Azotobacter*, *Derxia*). Most nitrogen fixers are bacteria, including cyanobacteria (B/G algae) and actinomycetes (*Frankia*).

It was long believed that there were no nitrogen-fixers among strains of the genus *Pseudomomas sensu stricto*. Indeed, consistent with this assumption, most of the strains described as putative nitrogen-fixing *Pseudomonas* were later reassigned to genera in the a- and b-Proteobacteria. It now seems that several strains unambiguously classified as true *Pseudomonas* spp. can be added to the list of nitrogen-fixers, on the basis of physiological properties, nitrogenase assays, phylogenetic studies and detection of nifH DNA by hybridization or PCR amplification. The ability to fix atmospheric nitrogen among the leguminous plants in association with rhizobia is found in several genera, and they account for most of the world's land-based BNF. At least one genus of non-legume that can nodulate with *Rhizobium* and *Parasponia* has been recognized, and others may be found. Although there is considerable diversity among the leguminous plants, the system for nitrogen fixation varies little. The taxonomy of the rhizobia—initially based on the host legume infected—is constantly being refined. There are presently four accepted genera: *Azorhizobium, Bradyrhizobium, Rhizobium,* and *Sinorhizobium*. *Rhizobiaceae* are in the alpha subgroup of the proteobacteria, and thus are related to other nitrogen fixers which do not interact with plants like photosynthetic bacteria (*Rhodospirillum rubrum*) or root colonizers which do not form differentiated structures on the host; like *Azospirillum*. The property of being able to fix nitrogen symbiotically with the formation of differentiated structures is found in two major groups of microbes not phylogenetically related: *Frankia* (among the Gram positive Thallobacteria), and rhizobia (in the alpha subgroup of Proteobacteria), and also with Cyanobacteria.

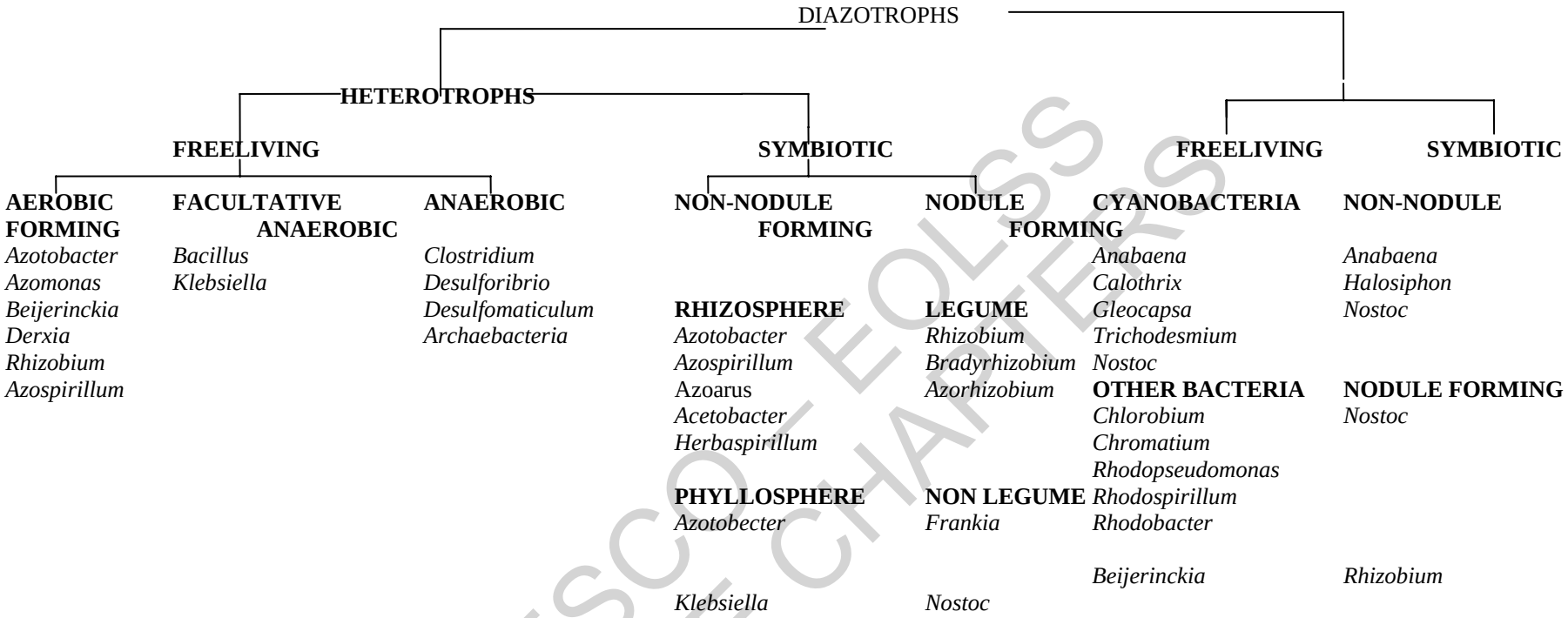

Table 2:. The Primary nitrogen-fixing genera.

In many ecosystems, non-leguminous plants with symbiotically associated actinorhizal micro-organisms (*Frankia*) fix substantial amounts of nitrogen. Other symbioses include those of termite with nitrogen-fixing bacteria located in their rear gut. Of some 50 bacterial species recognizable as distinct morphological types in the gut, about 40 were found to be nitrogen fixing. These bacteria expressed nitrogenase even in the presence of greater than 5mM ammonia in the hindgut, only switching off its expression when exposed to pure ammonia gas.

Nitrogen fixing systems are typical in pioneer communities, in harsh environments and in nitrogen-deficient soils. Thus acacias are prominent in forest being re-established after clearing of timber. Casuarinas thrive in similar habitats, and when well adapted to ecosystems their ability to fix nitrogen gives them a competitive edge. Once ecosystems become enriched with nitrogen, this competitive advantage may be lost and the nitrogen-fixing species are likely to decline in significance. The breadth of ecosites where BNF has been recognized is indicated in Table 3.

Ecosystem	Nature of BNF	Type	Occurrence/usage
Natural	Symbiotic	Root and stem Nodule *Rhizobium, Frankia* Mosses, Lichens, Pteridophytes Insects *Gunnera-Nostoc*	Legumes Actinorhizal trees/shrubs Soil, rock and tree surfaces Gut of termites Base of leaves, Cycad roots
	Non-symbiotic	Free-living saprophytes Photosynthetic *Anabaena, Nostoc* etc. *Rhodospirillum* etc	Soil and plant root rhizosphere bacteria on litters On plant surfaces Cyanobacteria (B/G algae Aquatic and marine bacteria
Agricultural/Forestry	Symbiotic	Nodulated legumes Actinorhizal angiosperms	Annual crops, perennial crops, Rotations, green manure Plantation systems Pioneer uses, etc
	Non-symbiotic	Miscellaneous symbiotic As above	*Azolla*, sugar cane etc Rice paddies

Table 3. Distribution of Nitrogen-fixing systems.

Bacterial system	Fixation rates (kg ha^{-1} per year)
Legume/*Rhizobium* symbioses	24-584
Azolla/*Anabaena* symbioses	45-450
Frankia symbioses	2-362
Free-living and associative bacteria	Trace-36

Table 4: Reported ranges of BNF by different bacterial systems in tropical soils.

The magnitude of the biologically fixed nitrogen through some of these processes is shown in table 4.

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Bibliography

Bishop P. E., Jarlenski D. M. L., and Hetherington D. R. (1980). Evidence for an alternative nitrogen-fixing system in *A. vinelandii*. *Proceedings of the National Academy of Sciences 77:7342-7346.* [This paper reports on the existence of the first alternative nitrogenase system in *Azotobacter vinelandii*.]

Brady N. C. and Weil R. R. (2000). Nitrogen and sulfur economy of soils. *Elements of the Nature and Properties of Soils.* pp. 358–390. Prentice Hall. [This book explores the principles and properties of soils and their physical, chemical and biological characteristics.]

Carnahan J. E., Mortenson L. E., Mower H. E. and Castle J. E (1960). Nitrogen fixation in cell-free extracts of *Cl. pasterianum*. *Biochem. Biophys. Acta* **44,** 520–535. [This is the first report of the isolation of nitrogenase enzyme under cell-free environment.]

Chisnell J. R., Premakumar R., and Bishop P. E. (1988). Purification of a second alternative nitrogenase from a *nifHDK* deletion strain of *A.vinelandii*. *Journal of Bacteriology* **170**, 27–33 [This paper reports on the existence of the second alternative nitrogenase system in *Azotobacter vinelandii*.]

Clarke, T.A., Maritano, S., and Eady, R.R. 2000. Formation of a tight 1:1 complex of Clostridium pasteurianum Fe protein-*Azotobacter vinelandii*MoFeprotein: evidence for long-range interactions between the Fe protein binding sites during catalytic hydrogen evolution. *Biochemistry* **39:**11434–11440.

Elkan G. H. (1992) BNF-systems in tropical-ecosystems: an overview. *BNF and Sustainability in Tropical Agricultural* (Eds. K. Mulongoy, M. Gueye, and D. S. C. Spencer). Wiley, New York. pp. 27–40. [This book is a Proceedings of the Fourth International Conference of the African Association of BNF]

Kahindi J. H–P., Woomer P. L., George T, de Souza Moreira M., Karanja N. K. and Giller K. E. (1995). Agricultural intensification, soil biodiversity and Ecosystems Function: The Role of Nitrogen Fixation Bacteria. *Journal of Soil Applied Ecology* **6** (part 1) 55–76. [Detailed review of the biological nitrogen fixing systems.]

Karanja N., Freire J., Gueye M., and DaSilva E. (2000). MIRCEN Networking: Capacity-Building and BNF Technology Transfer in Africa and Latin America. *AgbioTechnet Conference*, CABI, March 2000: Chichester, New York, Brisbane, Toronto, Singapore , John Wiley[Detailed account of the three BNF-MIRCENs in Africa and South America and their achievements.]

Igarashi R.Y. and Seefeldt, L.*C (2003)* Nitrogen Fixation: The Mechanism of the Mo-Dependent Nitrogenase. *Critical Reviews in Biochemistry and Molecular Biology*, 38:351–384 [This review focuses on recent developments elucidating the mechanism of the Mo-dependent nitrogenase].

Kim J. and Rees D.C. (1992). Structural models for the metal centers in the nitrogenase MoFe protein. *Science* **257**, 1677–1682 [This is the first structural model of the interaction of the dinitrogen molecule with the nitrogenase enzyme.]

Maritano, S., Fairhurst, S.A., and Eady, R.R. 2001. Longrange interactions between the Fe protein binding sites of the MoFe protein of nitrogenase. *J Bio Inorg Chem* **2001:**590–600.

Mullin B. (1997). *Frankia* - based systems*BNF: The Global Challenge and Future Needs.* pp. 59–65. *Rockefeller Foundation Bellagio Conference* Lake Como, Italy, April 8–12, 1997.] [This is a position paper on BNF]

Sprent J. I. and Sprent P. (1990). *Nitrogen Fixing Organisms*. 256 pp. Chapman and Hall publishers. [This book provides a wide ranging, lucid and up to date introduction to BNF.]

Woomer P. , Singleton P. W. and Bohlool B. B. (1988). Ecological indicators of native rhizobia in tropical soils. *Applied Environmental Microbiology* **54**, 1112–1116. [Gives an account of factors influencing populations of indigenous rhizobia in soil.]

Young J. P. W. (1994). All those new names: an overview of the molecular phylogeny of the plant-associated bacteria. *Advances in Molecular Genetics of Plant-Microbe Interactions* vol. 3 (Eds M. J. Daniels. Dordrecht; Kluwer pp.73–80. [This is a comprehensive overview of the taxonomy of the rhizobia.]

Biographical Sketches

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