BIOLOGICAL NITROGEN FIXATION WITH EMPHASIS ON LEGUMES

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Summary

Biological nitrogen fixation is the biological reduction of dinitrogen gas to ammonia under normal temperature and pressure. The capacity to perform the reaction is an ancient feature of prokaryotic microorganisms. Eukaryotes get access to biologically fixed combined nitrogen by forming symbioses with prokaryotic nitrogen fixers. The best-known nitrogen-fixing symbiotic system is the legume root nodule. The biochemical signal exchange leading to the formation of the nodule is fairly well understood, but the diversity of nitrogen-fixing legumes is less known.

The agronomic utilization of biological nitrogen fixation has a long history. In forestry and agroforestry, nitrogen-fixing multipurpose trees are becoming important and more research is needed to optimize their use. The field has a long tradition of applied biotechnology using legume inoculation for increased nitrogen fixation and yields.

The new biological era of genomics will be beneficial for further studies of the diversity of nitrogen-fixing organisms, their biochemical interaction and ecology and will hopefully lead to increased use of this natural resource for sustainable plant and animal production.

1. Nitrogen-Fixing Organisms

Biological nitrogen fixation is a natural process in which atmospheric dinitrogen gas (N_2) is converted to ammonia:

$N_2 + 4H_2 \Leftrightarrow 2NH_3 + H_2$

The reaction takes place under normal temperature and pressure and is catalyzed by the enzyme nitrogenase. This is a remarkable achievement, since industrial reduction of dinitrogen to ammonia requires high temperatures and pressures, consuming large amounts of energy, about 1.5 kg oil per 1 kg fertilizer nitrogen. Also the biological process is energy demanding, consuming 16–24 mol of ATP for the reduction of one mole of dinitrogen.

Nitrogenase is in fact an enzyme complex consisting of two subunits, dinitrogenase and dinitrogenase reductase. Both enzymes contain iron, and dinitrogenase contains molybdenum as well. The role of the dinitrogenase reductase is to supply electrons to the dinitrogenase, one at a time, each delivery being associated with the consumption of one ATP.

The genes encoding the nitrogenase complex are called *nif* genes, and they are remarkably conserved among nitrogen-fixing organisms. Because special machinery is required to perform the whole process, many more genes are needed for accessory functions. Their nature will however vary depending on the organism.

The nitrogenase enzyme is only synthesized by prokaryotes. It is found in both main domains of prokaryotes, in the Archaea and in the Bacteria. Thus, the capacity to reduce nitrogen gas is a widespread phenomenon, occurring in several main prokaryotic kingdoms.

Interestingly, no eukaryotes synthesize nitrogenase by themselves. In order to get access to biologically reduced nitrogen, some higher plants have developed a symbiotic relationship with nitrogen-fixing bacteria, in which they get access to bacterially reduced nitrogen.

Many leguminous plant species (family Fabacae) can enter a symbiotic relationship with root-nodule bacteria, collectively called rhizobia (bacterial genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium*). Among about 20 000 legume species examined, several thousand carry root nodules. Only one non-legume, the woody plant *Parasponia* sp., can be nodulated by rhizobia and utilize the nitrogen fixed by the bacteria. The rhizobia are gram-negative, belonging to the large and important alpha-proteobacterial kingdom. Recently, bacteria belonging to the genus *Methylobacterium* and even to the beta-proteobacterial *Ralstonia* and *Burkholderia* were found to nodulate legumes. Gram-positive, streptomycete-like bacteria from the genus *Frankia*, are another group capable of symbiotic nitrogen fixation. *Frankia*-strains infect a wide variety of plants from the Rosid I lineage of angiosperms, such as *Alnus*, *Myrica*, and *Casuarina*.

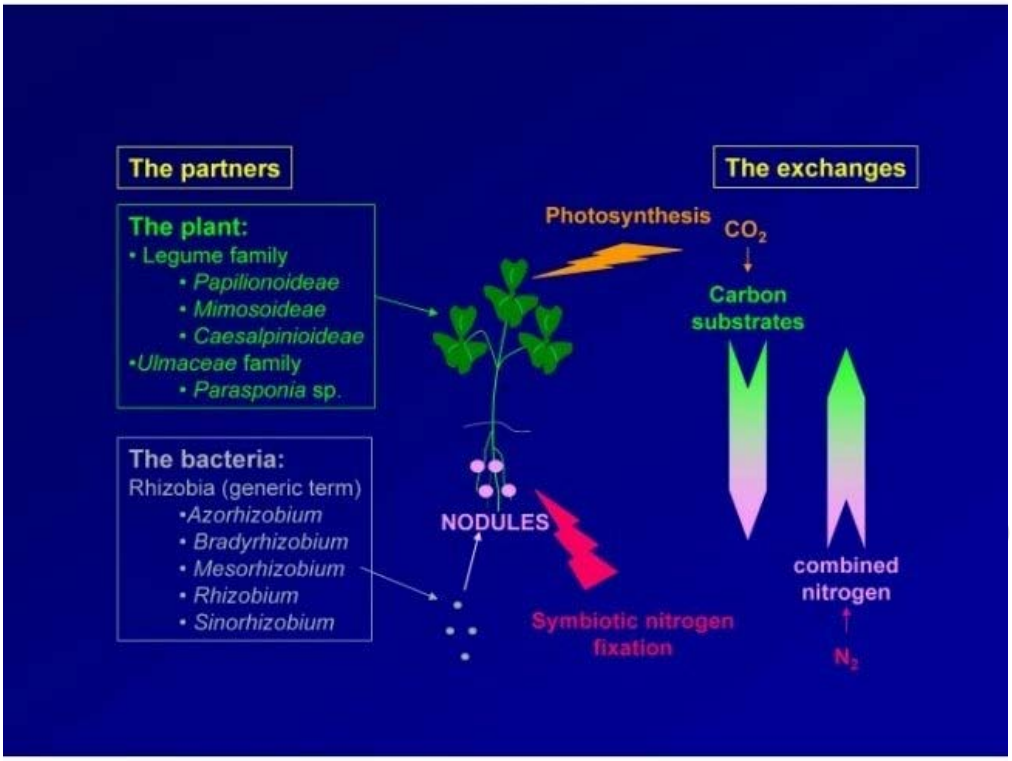

Figure 1. Exchanges between the legume host and its symbiotic partner. Source: courtesy Gilles Lortet.

Cyanobacteria form another group of important nitrogen-fixing prokaryotes. Nitrogen fixation has been detected both in unicellular and in multicellular cyanobacteria. Certain cyanobacteria can enter symbiotic relationships with plants. *Anabaena azollae* fixes nitrogen symbiotically with the water fern *Azolla*, which is important in rice fields. *Nostoc* species can infect special organs—glands—on plants belonging to the genus *Gunnera*, and nitrogen-fixing *Nostoc* species are also found in the root cavities of cycads, primitive plants growing in the tropics.

Examples of free-living nitrogen-fixing bacteria are the chemo-organotrophic *Azotobacter, Beijerinckia, Klebsiella, Paenibacillus, Desulfovibrio* and *Clostridium,* the phototrophic cyanobacteria (some), *Rhodospirillum* and *Rhodobacter*, and chemolithotrophic *Alcaligenes* and *Thiobacillus* (some). Among the Archaea, *Methanosarcina* and *Methanococcus* can fix nitrogen.

The reduction of dinitrogen is an anaerobic but energy-requiring process. Since aerobic respiration is the form of energy metabolism that generates the largest amounts of energy per substrate used, the nitrogen-fixing organisms face some problems in resolving the seeming paradox of generating large amounts of energy and reducing power for the nitrogenase enzyme. For anaerobic bacteria, such as *Clostridium,* this is no problem. In nitrogen-fixing root nodules of legumes, the oxygen transporter leghemoglobin ensures oxygen transport for the respiration machinery, while maintaining a low partial pressure of free oxygen that could otherwise damage the nitrogenase. In nodules on alder (*Alnus*) the nitrogen-fixing bacteria are enclosed by

thick-walled, plant-derived vesicles, which serve as oxygen barriers. Cyanobacteria, which generate energy but also oxygen through photosynthesis, have resolved the problem by separating nitrogen fixation from photosynthesis either temporally or spatially. Some cyanobacteria thus photosynthesize during the day and fix nitrogen during the night. Others have specialized cells, heterocytes, which do not photosynthesize but only fix nitrogen.

Streptomyces thermoautotrophicus is different from other nitrogen fixers. This gram-positive prokaryote lives in composts and burning charcoal piles and can reduce dinitrogen by employing an enzyme system that is insensitive to oxygen. It also requires less energy than the classical nitrogenase.

2. Importance of Nitrogen Fixation

2.1. Inputs to the Ecosystems

The amount of nitrogen contributed to the biosphere through biological nitrogen fixation has been estimated to range from 63 x 10^6 to 175 x 10^6 tonnes per year. Symbiotic nitrogen fixation in legumes contributes approximately 30% to this amount. However, leguminous nitrogen fixation is the most efficient system, the mean yearly fixation rate being 55–140 kg nitrogen per hectare, compared with 0.3–30 kg per hectare for other nitrogen-fixing biological systems. These figures may change over time, when, for example, the contribution of cyanobacterial nitrogen fixation to the oceans can be better estimated.

In the United Kingdom it was estimated that white clover (*Trifolium repens*) in mixture with grasses yearly fixed up to 280 kg nitrogen per hectare, and that up to 45% of nitrogen fertilizer was saved, when white clover was introduced into a sward. In 1980 the mean rate of chemical fertilizer application in Finland was 83 kg of nitrogen per hectare agricultural soil; 15 kg was applied as manure and approximately 20 kg added through precipitation and biological nitrogen fixation. The proportion of clover (*Trifolium* sp.), the main nitrogen-fixing legume in Finland, in swards and leys was less than 5%. The situation today (2002) is probably quite similar. Organic farmers are dependent on biological nitrogen fixation, so the proportion of those (now 2% in Finland) will decisively influence the figures in the future.

2.2. Methods to Measure Nitrogen Fixation

There are several direct and indirect ways to measure biological nitrogen fixation. Even though they all suffer from uncertainties and drawbacks, they are versatile tools for different systems and conditions.

The acetylene reduction assay is a convenient, indirect method to assay the activity of the nitrogenase enzyme system. It was developed based on the fact that the nitrogenase system can reduce several alternative substrates, such as acetylene (C_2H_2), which is reduced to ethylene (C_2H_4). The assay is performed by incubating the nitrogen-fixing organism (a bacterial culture, a soil sample, or root nodules) in a gas-tight vessel and adding 5–10% of acetylene to the vessel atmosphere. At this acetylene concentration the

nitrogenase system stops reducing dinitrogen and turns completely into an acetylene-reducing system, because the affinity of the enzyme for acetylene is very high. Since the product of the reaction is not required by the organisms, it is released to the atmosphere of the vessel. By sampling at regular intervals and analyzing the gas samples by gas chromatography, the operator can estimate the activity of the nitrogenase. The assay is versatile and is used to detect nitrogenase activity both in biochemical laboratory experiments with purified nitrogenase and in field experiments with intact legume plants. Nitrogenase activity in forage legumes (*Trifolium*, *Medicago*, and *Galega* species) growing in northern temperate conditions was, for example, detected by acetylene reduction at soil and air temperatures close to zero degrees Celsius.

Because the acetylene reduction assay is indirect, the conversion of the amount of acetylene reduced to the amount of nitrogen fixed is problematic. In order to get proper estimates of the amounts of biologically fixed nitrogen, more direct methods should be used.

In agriculture, a cheap, direct method is to determine the amount of nitrogen in the crop yield. If a crop to which no nitrogen fertilizer has been applied yields 100 kg of nitrogen, the nitrogen must have come from biological fixation and soil. By comparison with the nitrogen content of a non-fixing reference plant, the difference in nitrogen content can be assumed to come from biological nitrogen fixation. If the concentration of mineral nitrogen in soil is estimated at the beginning of the growing season and at harvest time, the estimates can be made more accurate. Another way of measuring the amounts of nitrogen fixed is to apply different levels of nitrogen fertilizer and then compare the harvests for nitrogen content.

Isotopic techniques offer versatile tools both for the direct estimation of biological nitrogen fixation and for studying nitrogen cycling in ecosystems. The stable isotope ^{15}N is used most often, since the radioactive isotope ^{13}N has a half-life of only 10.5 minutes. The most straightforward method is to incubate a nitrogen-fixing organism in an atmosphere enriched with ^{15}N, followed by subsequent mass-spectrometric analysis of the biological material for enrichment of ^{15}N. This method is fairly expensive and cannot be used *in situ* (in the field). Less isotope is required when using the isotope dilution method, in which ^{15}N is applied as fertilizer to a nitrogen-fixing crop. This method can also be used in the field. Depending on its nitrogen nutrition (fertilizer versus atmospheric nitrogen), more or less isotope will be enriched in the plant tissues, assuming that the plant always takes up some nitrogen from soil. For estimation of the amounts of nitrogen derived from the atmosphere, a non-fixing control plant must be used. This plant should ideally behave exactly like the fixing plant, except for nitrogen fixation.

None of the methods described is well suited for the assessment of biological nitrogen fixation in wild plants. For that purpose the natural abundance method has been developed. This method relies on the fact that biological systems, such as the nitrogenase enzyme, discriminate between isotopes in favor of lighter ones. This means that the nitrogenase prefers the more abundant ^{14}N isotope to the heavier ^{15}N. A nitrogen-fixing plant will consequently be enriched for ^{14}N over ^{15}N. This non-destructive method requires careful calibration to be quantitative, but will at least work

satisfactorily at a fixing/non-fixing level. It is a good method for the estimation of nitrogen fixation in natural or organic farming ecosystems, and for trees it is often the only alternative.

3. The *Rhizobium*–Legume Symbiosis

3.1. The Rhizobia

The rhizobia or root-nodule bacteria are gram-negative soil bacteria belonging to the alpha-proteobacteria. They are characterized by the capacity to form root or stem nodules on leguminous plants. They belong to several related bacterial genera: *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium*. Recently even strains belonging to the genus *Methylobacterium, Ralstonia* and *Burkholderia* were found to be nodule-forming.

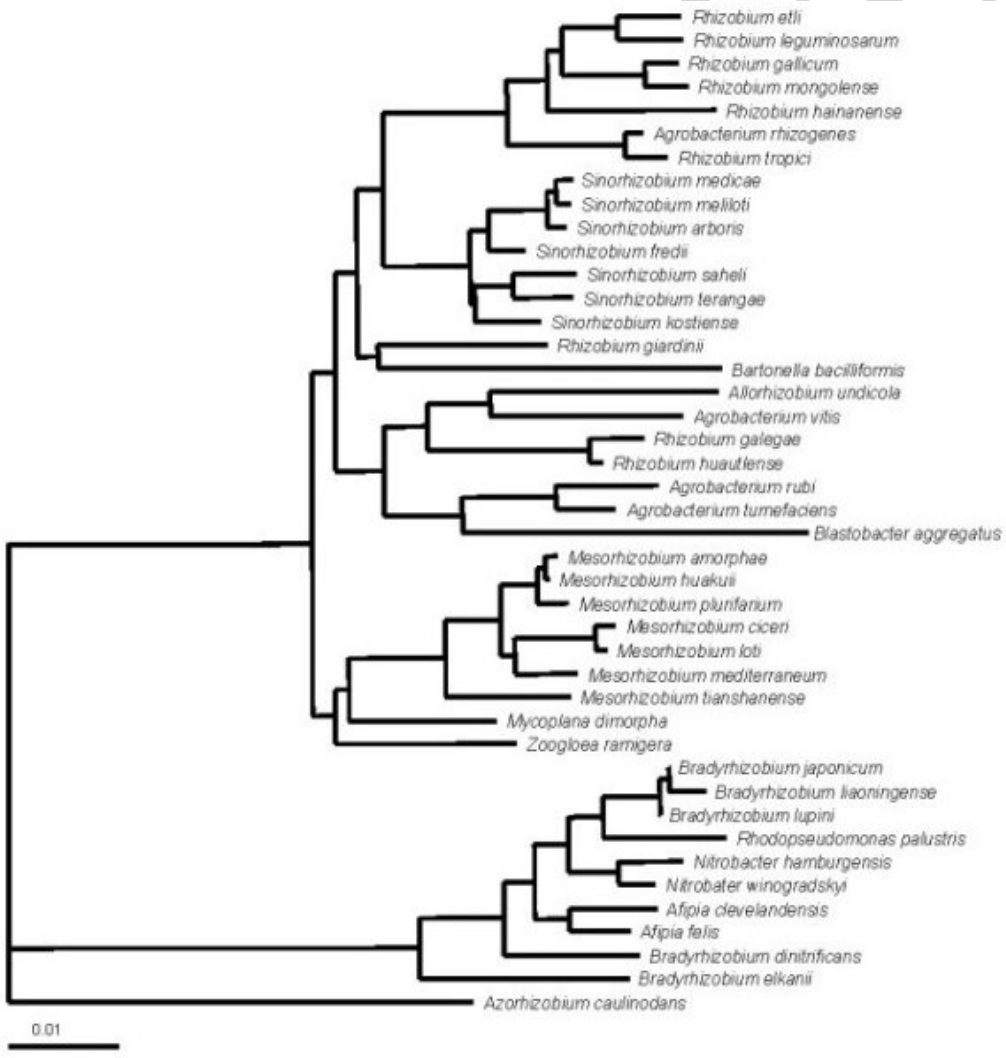

Figure 2. A phylogenetic tree of rhizobia and related bacteria based on 16S ribosomal sequences. Source: courtesy Leena Suominen and Zewdu Terefework.

Figure 2 shows a phylogenetic tree based on 16S ribosomal sequences. It can be seen that the rhizobia are dispersed between bacteria with other metabolic functions, such as photosynthesis (*Rhodopseudmonas*), nitrification (*Nitrobacter),* and pathogenicity (*Agrobacterium*).

The rhizobia can also be classified according to host range. Table 1 shows the currently recognized species with representative host plants. For reasons that are not yet fully understood, some rhizobia have a very narrow host range, whereas that of others is extremely broad. The phylogeny and the host specificity are not correlated. The reason for this is that the rhizobia are equipped for two different kinds of life, one in soil and another inside the plant. Genes required for general metabolic functions and for a life in soil are those that are used for classifying rhizobia taxonomically and phylogenetically. However, it is now generally accepted that the rhizobia acquired the genes needed for interaction with plants via lateral transfer.

Thus the evolutionary history of these genes is different from that of ribosomal sequences used to construct the phylogenies of the bacteria. Sometimes the same species contains groups of strains with a clearly divergent host range for nodulation and/or fixation. Strains with the same host range are then conveniently assigned biovar status. For example *Rhizobium leguminosarum* has three biovars, viciae (hosts *Vicia, Pisum, Lathyrus*), trifolii (hosts *Trifolium*), and phaseoli (hosts *Phaseolus*), distinguished by their nodulation capacity. *Rhizobium galegae* has two biovars, orientalis and officinalis, which are distinguished based on their host specificity for nitrogen fixation.

Rhizobial species	**Host plants**
Allorhizobium	
A. undicola	Neptunia
Azorhizobium	
A. caulinodans	Sesbania (stem nodules)
Bradyrhizobium	
B. japonicum	Glycine, Macroptilium, Vigna, Arachis, many wild legumes
B. elkanii	Glycine, Macroptilium, Vigna
B. liaoningense	Glycine
Mesorhizobium	
M. amorphae	Amorpha
M. ciceri	Cicer
M. huakuii	Astragalus
M. loti	Lotus, Anthyllis, Lupinus
M. mediterraneum	Cicer
M. plurifarium	Acacia, Prosopis
M. tianshanense	Glycyrrhiza
Rhizobium	

R. etli	Phaseolus
R. gallicum	Phaseolus
R. giardinii	Phaseolus
R. galegae	Galega
R. hainanense	Desmodium
R. huautlense	Sesbania
R. leguminosarum	Pisum, Vicia, Trifolium, Phaseolus
R. mongolense	Medicago
R. tropici	Phaseolus, Leucaena, Macroptilium
Sinorhizobium	
S. arboris	Acacia, Prosopis
S. fredii	Glycine, Vigna
S. kostiense	Acacia, Prosopis
S. medicae	Medicago, Melilotus
S. meliloti	Medicago
S. saheli	Sesbania
S. terangae	Acacia, Sesbania

Note: For species with broad host range only some hosts have been indicated.
Strains belonging to *Methylobacterium, Ralstonia* and *Burkholderia* have also been found to nodulate

Table 1. Rhizobial species and selected host plants

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Biographical Sketch

Associate professor **Kristina Lindström** received her master and doctoral degrees in microbiology at the University of Helsinki, Faculty of Agriculture and Forestry. Her doctoral thesis was entitled *Ecological studies of symbiotic nitrogen fixation in temperate forage legumes* (1985). She spent 1981–1982 as an OECD fellow at Massey University in New Zealand, studying Rhizobium taxonomy, and in 1983–1984 she was a visiting scientist in Professor Fred Ausubel's laboratory at Harvard University and Harvard Medical School in Boston, USA, learning molecular methods with Rhizobium. Her research has since then dealt with various aspects of Rhizobium taxonomy, diversity, molecular biology, molecular ecology, and evolution. Her group has described new rhizobial species and has been actively developing methods for detection of DNA from soil. Molecular fingerprinting methods and reporter and marker genes are now used to study rhizobial ecology and diversity with bacteria from trees in Africa, from peanut in China and from temperate forage legumes, including risk assessment of genetically modified bacteria released into the environment.

Associate professor Lindström is the secretary of the International Subcommittee on Agrobacterium and Rhizobium of the International Committee on Systematics of Procaryotes. She is the curator of the HAMBI microbial culture collection at the University of Helsinki. She has a keen interest in university teaching at all levels, being the organizer of several international training courses and the supervisor of both domestic students and students from abroad, for example, China.