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CHAPTER 1.24

# Dinitrogen-Fixing Prokaryotes

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## Introduction

Dinitrogen fixation, the biocatalytic conversion of gaseous nitrogen ($N_2$) to ammonium, is an exclusive property of prokaryotes. The enzymes responsible for this reaction are nitrogenases. Proof that bacteria associated with leguminous plants can fix atmospheric $N_2$ (making it available to the plants for growth) was first reported in 1888 (reviewed in Quispel, 1988). Nitrogen fixation is the most important way $N_2$ from air enters biological systems and therefore it is a key step in the nitrogen cycle. From a practical point of view, the importance of the process rests with its ability to reduce the chemical fertilization of crops, even under conditions of environmental stress (Bordeleau and Prévost, 1994; Zahran, 1999). Indeed, agronomically important crops such as soybean, alfalfa, pea, clover and bean obtain substantial amounts of their nitrogen from bacterial $N_2$ fixation. One of the long-term goals of $N_2$ fixation research is to select or engineer major cereal crops such as rice, maize and sugarcane so they can satisfy the bulk of their nitrogen requirements, either indirectly by association with $N_2$-fixing bacteria, or directly by insertion of $N_2$-fixing genes into the plant.

Many diazotrophs (*di* = two, *azo*te = nitrogen; *trophs* = eaters: dinitrogen fixers) are found to be associated with the roots of plants where they exchange fixed nitrogen for the products of photosynthesis. Plants associated with $N_2$ fixers can grow in very poor soils and swamps (Koponen et al., 2003) and be used successfully for soil remediation.

Nowadays industrial fixation of atmospheric $N_2$ exceeds the amount estimated to be produced by biological nitrogen fixation each year (Karl et al., 2002) and increased nitrogen (N) deposition seems to be responsible for loss of biodiversity and plant species extinction (Stevens et al., 2004). Biological $N_2$ fixation is still the main source of nitrogen in soil, marine environments such as oligotrophic oceanic waters (where dissolved fixed-nitrogen content is extremely low; Zehr et al., 1998; Staal et al., 2003), subtropical and tropical open ocean habitats (Karl et al., 2002), and hydrothermal vent ecosystems (Mehta et al., 2003). $N_2$ fixation in coastal marine environments may diminish because of habitat destruction and eutrophication (Karl et al., 2002). Dinitrogen fixation may be a major nitrogen source for supporting primary and secondary production of biomass in Antarctic freshwater and soil habitats (Olson et al., 1998) and has been reported to occur in moss carpets of boreal forests (DeLuca et al., 2002) and in woody debris (Hicks et al., 2003). Dinitrogen fixation by bacteria inside insect gut helps to compensate termites for their nitrogen-poor diet (Kudo et al., 1998; Nardi et al., 2002).

$N_2$-fixing prokaryotes inhabit a wide range of exterior environments (including soils, seas, and the oceans) and interior environments (including insects, cow rumena, human intestines, and feces; Bergersen and Hipsley, 1970), and even printing machines and paper-making chemicals (Vaisanen et al., 1998). Nevertheless, the presence of a $N_2$-fixing bacterium is not evidence for the occurrence of $N_2$ fixation. On the basis of N balance analyses, $N_2$ fixation seemed to account for excess N in humans with a low N diet, and N-fixing bacteria were obtained from their guts (Bergersen and Hipsley, 1970; Oomen and Corden, 1970).

Dinitrogen fixers are encountered in Bacteria and in some groups of Archaea. The number of nitrogen-fixing phyla or lineages within the domain Bacteria increased from 5 to 6 when nitrogen-fixing bacteria were discovered within the Spirochaetes (Lilburn et al., 2001). The inventory of the phyla containing nitrogen-fixing bacteria is probably still far from complete but enlarging, as with the report of a strain of *Verrucomicrobium* that is reported to have nitrogen fixation genes (Rodrigues et al., 2004). Lists of $N_2$-fixing prokaryotes have been published (Young, 1992; Phillips and Martínez-Romero, 2000), and new nitrogen-fixing species are continuously being described (Chen et al., 2001; Moulin et al., 2001; Distel et al., 2002; Von der Weid et al., 2002; Bianciotto et al., 2003; Rosenblueth et al., 2004). Nevertheless knowledge of

$N_2$ fixers is limited, and some not yet identified $N_2$ fixers could be found among the novel bacterial divisions that are mostly unculturable (Rappé and Giovannoni, 2003). The distribution of $N_2$ fixers among the prokaryotes is patchy (Young, 1992). They constitute restricted groups within larger bacterial clusters. The existence of non-fixers that are closely related to fixers has been explained by the loss of $N_2$ fixation genes or by the lateral transfer of these genes among bacterial lineages (Normand and Bousquet, 1989; Vermeiren et al., 1999). Nitrogen fixation is an energy costly process, which may explain why nitrogen fixation was lost in many bacterial lineages when not needed. The possession of $N_2$-fixing genes does not confer a selective advantage to bacteria in nitrogen-rich environments, as is the case where fixed nitrogen is added to agricultural fields. Application of ammonium sulfate reduced the number of *Azotobacter* in the plant rhizosphere, and when compared with plants fertilized with both nitrogen and phosphorus, maize treated with phosphate alone had increased nitrogenase activity (Döbereiner, 1974).

Similarly, very few or no *Gluconacetobacter diazotrophicus* microorganisms were isolated from sugarcane plants from heavily fertilized areas (Fuentes-Ramírez et al., 1993; Muthukumarasamy et al., 1999), and, perhaps as a result of chemical nitrogen fertilization, the bacterial population had very limited genetic diversity (Caballero-Mellado and Martínez-Romero, 1994; Caballero-Mellado et al., 1995). Subsequently, sugarcane colonization by *A. diazotrophicus* was found to be inhibited in plants supplied with chemical nitrogen fertilizer (Fuentes-Ramírez et al., 1999). Another effect of adding fixed nitrogen (diminished genetic diversity of *Rhizobium* from *Phaseolus vulgaris* bean nodules) was observed when the plants were treated with the recommended level of chemical nitrogen (Caballero-Mellado and Martínez-Romero, 1999).

The complete genome sequence of the Archaeon *Methylobacterium thermoautotrophicum* was reported in 1997 revealing the presence of *nif* genes (Smith et al., 1997), but $N_2$ fixation could not be demonstrated in this strain (Leigh et al., 2000). The sequences of the genomes of the legume-nodulating bacteria belonging to the genera of *Mesorhizobium* (Kaneko et al., 2000), *Sinorhizobium* (Galibert et al., 2001) and *Bradyrhizobium* (Kaneko et al., 2002) revealed contrasting chromosome sizes and highly diverging genomes. A common ancestor of *Mesorhizobium* and *Sinorhizobium* was deduced to exist nearly 400 million years ago (Morton, 2002). One of the most novel areas in nitrogen fixation research is genomics, and for sure many $N_2$-fixing bacteria will be used for the determination of their whole genome sequence in the near future. Post-genomic studies are already on course as well.

## Diazotroph Isolation and Conditions for $N_2$ Fixation

$N_2$-fixing bacteria are normally isolated in N-free media. Whether a microorganism is a $N_2$ fixer is not easy to determine. In the past, claims for many fixers were shown to be erroneous, mainly because fixers were recognized by their ability to grow in nitrogen-free media. However, traces of fixed nitrogen in the media sometimes accounted for the bacterial growth. At other times, oligotrophic bacteria and fungi, which can grow on nitrogen-free media, have been incorrectly reported to be $N_2$-fixing organisms. These microorganisms appear to meet their nitrogen requirements by scavenging atmospheric ammonia (Postgate, 1988). Photosynthetic bacteria have been known for more than 100 years, but the capacity of some of these bacteria to fix $N_2$ was not recognized until much later. Microorganisms may fix $N_2$ under special conditions that may not be readily provided in the laboratory. For example, nitrogenases are inactivated in the presence of oxygen, and different levels of oxygen seem to be optimal for different $N_2$-fixing organisms. Also, some bacteria (e.g., some *Clostridium*) fix $N_2$ only in the absence of oxygen. In other cases, fixation may require specific nutritional conditions or a differentiation process or both. A remarkable case is the differentiation process of *Rhizobium* to form $N_2$-fixing bacteroids (Bergersen, 1974; Glazebrook et al., 1993) inside plant root or stem nodules. *Bradyrhizobium* species can fix $N_2$ both in plant nodules and in vitro, when provided with succinic acid and a small amount of fixed nitrogen (Phillips, 1974). To fix $N_2$, bacteria belonging to the genus *Azoarcus* (obtained from Kallar grass and more recently also from rice plants) require proline, undergo differentiation, and form a structure called a "diazosome" (Karg and Reinhold-Hurek, 1996). Stimulated by plants, cyanobacteria differentiate into $N_2$-fixing heterocysts that protect nitrogenase from oxygen (Wolk, 1996). Light was found to induce circadian rhythms of $N_2$ fixation in the cyanobacterium *Synechococcus* (Chen et al., 1993). Wheat germ agglutinins were found to stimulate $N_2$ fixation by *Azospirillum*, and a putative receptor of this agglutinin was found in the *Azospirillum* capsule. The stimulus generated from the agglutinin-receptor interaction led to elevated transcription of both structural and regulatory nitrogen-fixation genes (Karpati et al., 1999).

## Methods for Detecting Nitrogen Fixation

The methods used to measure biological $N_2$ fixation include the quantification of the total nitrogen difference from Kjeldahl analysis, acetylene reduction, and $^{15}N$ incorporation or dilution. The acetylene reduction assay has been used for over 30 years to measure nitrogenase activity and as an indicator of $N_2$ fixation (Hardy et al., 1968). These methods have been used both in the laboratory and the field, and improvements of the methods especially for field evaluations have been proposed, including double labeling using $^{34}S$ as a control reference (Awonaike et al., 1993). The $^{15}N$-based techniques have been thoroughly reviewed (Bergersen, 1980; Hardarson and Danso, 1993).

Nitrogenases may reduce other substrates in addition to $N_2$ and this has been the basis for the acetylene reduction assay, which measures $N_2$ fixation activity indirectly. However, the nitrogenase described by Ribbe et al. (1997) does not have the ability to reduce acetylene. In *Paenibacillus*, $N_2$ fixation has been demonstrated in some cases by the increase in nitrogen measured by the microKjeldahl method but not by acetylene reduction (Achouak et al., 1999).

To circumvent the problems of estimating $N_2$ fixation under laboratory conditions, a strategy to detect nitrogenase genes has been successfully followed. This strategy was made possible by identification of conserved signatures (useful as primers for the synthesis of the nitrogenase reductase gene by means of polymerase chain reaction [PCR] amplification) in the structural *nif* gene sequences, namely *nifH*, found in many microorganisms (Dean and Jacobson, 1992; Ueda et al., 1995). In other cases, homologous or heterologous probes have been used in hybridization experiments to detect $N_2$ fixers. With some *nifH* primers containing conserved sequences, alternative nitrogenases may also be amplified but not the nitrogenase (superoxide) that is structurally unrelated to the classical nitrogenase (Ribbe et al., 1997). Thus a search for $N_2$-fixing organisms using a procedure based only on the classical *nifH* gene would be incomplete. Nevertheless, with nitrogenase DNA primers and PCR synthesis, novel $N_2$-fixing genes may be found. Eight *nifH* gene types corresponding mainly to those of diazotrophic Proteobacteria were detected in rice root from endophytic or rhizoplane-borne bacteria (Ueda et al., 1995). Remarkably, none of the sequences amplified corresponded to previously described *nifH* sequences. The nucleotide sequence of one of the types was found to resemble those of the *Azoarcus nif* genes. Some bacteria in the gut of termites also have *nifH* sequences similar to those obtained from rice roots (Ohkuma et al., 1999). *nif* genes were found in human and bovine treponemas (Lilburn et al., 2001) but not in the completely sequenced genomes of the spirochetes *Treponema pallidum* or *Borrelia burgdorferi*.

Few $N_2$-fixing organisms from the oceanic environment have been cultivated and it is estimated that less than 10% of marine diazotrophs are cultivable. Nevertheless, on the basis of the amplification of nitrogenase *nifH* genes, new $N_2$-fixing organisms have been detected in oligotrophic oceans. Nitrogenase genes characteristic of cyanobacteria and of Alpha- and Betaproteobacteria were obtained, whereas *nifH* sequences from samples associated with planktonic crustaceans were found to be clustered with the corresponding sequences from either sulfate reducers or clostridia (Zehr et al., 1998). Since knowledge of the nitrogenase gene diversity has improved (over 1500 sequences were available at the time this manuscript was being written), different sets of primers have been designed (Bürgmann et al., 2004) to better amplify *nifH* genes directly from DNA extracted from various samples including environmental samples. More diverse diazotrophic populations have been revealed with this approach than with classical microbiological techniques that require culturing of the bacteria (Zehr et al., 1998; Bürgmann et al., 2004).

A different method of $N_2$-fixation detection involves the growth of indicator non-$N_2$-fixing organisms in a co-culture with putative $N_2$-fixing bacteria. Such an approach has the additional advantage of identifying bacteria that not only fix $N_2$ but also can release fixed nitrogen into the environment and thereby have potential use in agriculture. *Gluconacetobacter diazotrophicus* (Yamada et al., 1997), a $N_2$-fixing isolate from sugarcane, was cultured with the yeast *Lipomyces kononenkoae* on nitrogen-free medium, and yeast growth was shown to be proportional to the amount of $N_2$ fixed (Cojho et al., 1993).

## Distribution of Dinitrogen-Fixing Ability among Prokaryotes

Archaea and Bacteria nitrogenases are phylogenetically related (Leigh, 2000), and supposedly the last common ancestor was a $N_2$-fixing organism (Fani et al., 1999). Alternatively, nitrogen fixation could have evolved in methanogenic archaea and subsequently transferred into the bacterial domain (Raymond et al., 2004). Nowadays, only 6 out of 53 currently identifiable major lineages or phyla within the domain Bacteria

have nitrogen-fixing members, namely: Proteobacteria, cyanobacteria, Chlorobi (green nonsulfur), spirochetes and the Gram-positives (Firmicutes and Actinobacteria; Fig. 1).

Dinitrogen-fixing organisms have an advantage over non-fixers in $N_2$-deficient but not in $N_2$-sufficient environments where the $N_2$ fixers are readily outcompeted by the bulk of microorganisms. The *nif* genes may be expected to disappear from bacteria that become permanent inhabitants of environments with available fixed $N_2$; this may explain why some non-$N_2$ fixers emerged and are closely related to $N_2$ fixers in phylogenetic trees of bacteria. Even within species of $N_2$ fixers, some strains do not fix $N_2$ perhaps because of the loss of this unique capacity, as is evident in *Azotobacter*, *Beijerinckia* (Ruinen, 1974) and *Frankia* (Normand et al., 1996). In *Rhizobium*, *nif* genes and genes for nodule formation may be easily lost concomitantly with the symbiotic plasmid (Segovia et al., 1991). Similarly, nonsymbiotic *Mesorhizobium* strains are found in nature that lack a symbiotic island (Sullivan et al., 1996). $N_2$-fixing species seem to be dominant in Rhodospirillaceae (Madigan et al., 1984), and within the methanogens (in Archaea), nitrogen fixation is widespread (Leigh, 2000). While all *Klebsiella variicola* isolates were $N_2$-fixing bacteria (Rosenblueth et al., 2004), only 10% of its closest relatives (*K. pneumoniae* from clinical specimens) had this capacity (Martńez et al., 2004).

The $N_2$-fixing capability is unevenly distributed throughout prokaryotic taxa, and $N_2$-fixing bacteria are in restricted clusters among species of non-$N_2$-fixing bacteria. Only a subset of cyanobacterial species are able to fix $N_2$. *Gluconacetobacter diazotrophicus* and a couple of other $N_2$-fixing species are the only diazotrophs in a larger group comprising *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* (Fuentes-Ramírez et al., 2001). Similarly, among aerobic endospore-forming Firmicutes (Gram-positive

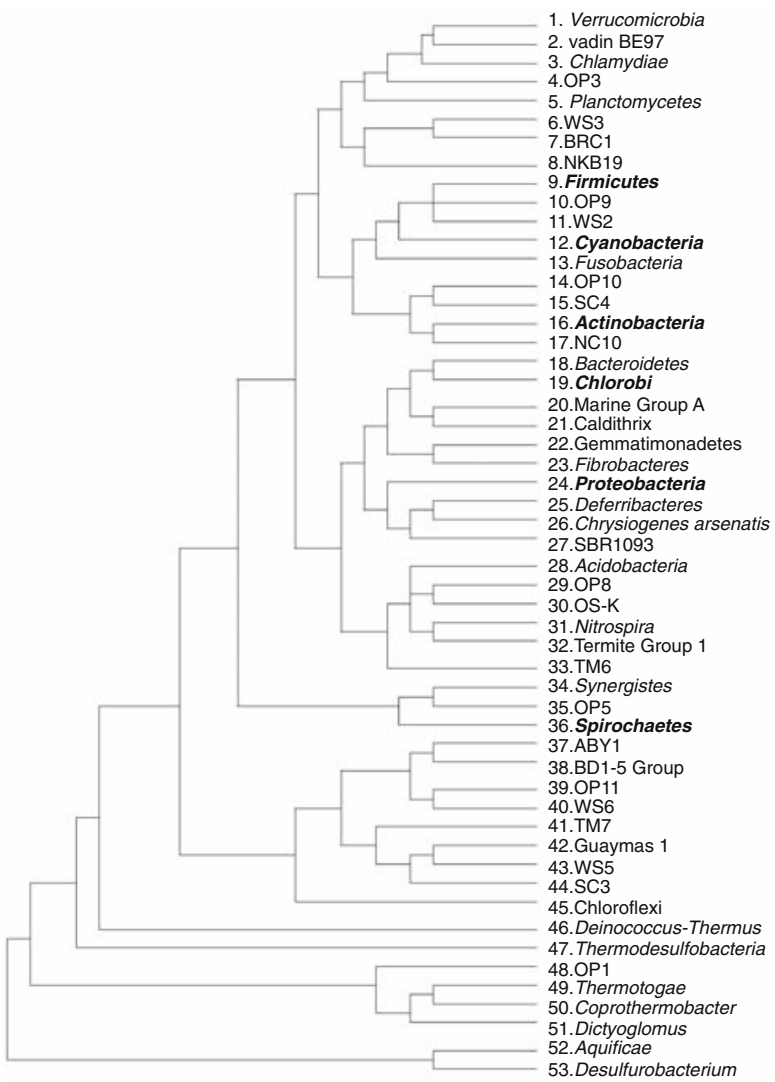


Fig. 1. Relatedness of *nifH* genes from different organisms according to DNA sequence (after Hurek et al., 1997a; Ueda et al., 1995; Young et al., 1992; Zehr et al., 1995). In parentheses, the Proteobacteria subclass.

bacteria), $N_2$ fixers are encountered mainly in a discrete group (defined by cluster analysis from 16S rRNA gene sequences) corresponding to *Paenibacillus* (Achouak et al., 1999). Among the actinomycetes, $N_2$-fixing *Frankia*, represented by a diversity of phenotypes from different habitats, are grouped by their 16S rRNA gene sequences (Normand et al., 1996). In Archaea, $N_2$-fixing organisms are found in the methanogen group and in the halophile group within the Euryarchaeota but not in the sulfur-dependent Crenarchaeota (Young, 1992).

*Pseudomonas* spp. were considered unable to fix $N_2$, but recently new isolates have been recognized as $N_2$ fixers. Some isolates, closely related to fluorescent pseudomonads, possess in addition to the FeMo nitrogenase an alternative molybdenum-independent nitrogenase (Loveless et al., 1999; Saah and Bishop, 1999). Dinitrogen-fixing *Pseudomonas stutzeri*, (previously designated *Alcaligenes faecalis*) (Vermeiren et al., 1999), is widely used as a rice inoculant in China (Qui et al., 1981). Following rice inoculation, *P. stutzeri* aggressively colonize the roots, and the *nifH* gene is expressed in these root-associated bacteria (Vermeiren et al., 1998). Other reports list different $N_2$-fixing *Pseudomonas* species that have been isolated from sorghum in Germany (Krotzky and Werner, 1987), from *Capparis* in Spain (Andrade et al., 1997), and from *Deschampsia caespitosa* in Finland (Haahtela et al., 1983). The sporadic occurrence of *nif* genes in *Pseudomonas* may be explained by the acquisition of these genes by lateral transfer (Vermeiren et al., 1999). *Pseudomonas stutzeri* strains are known to be naturally competent for DNA uptake (Lorenz and Wackernagel, 1990). Other *nifH* gene sequences obtained from rice-associated bacteria were in the same cluster as the *P. stutzeri nifH* gene (Ueda et al., 1995; Vermeiren et al., 1999).

The phylogenetic relationship of $N_2$-fixing organisms inferred from the comparative analysis of *nif* and 16S rRNA gene sequences led Hennecke et al. (1985) to propose that the *nifH* genes may have evolved in the same way as the organisms that harbor them; a similar conclusion was obtained by Young (1992) from the analysis of a larger number of diazotrophs. Ueda et al. (1995) and Zehr et al. (1995), using different reconstruction methods, reported *nifH* gene phylogenies in general agreement with the phylogenetic relationships derived from 16S rRNA gene sequences, with some exceptions. A more recent comparison of *nif*H and 16S rRNA phylogenies has been performed with a very short fragment of the *nifH* gene. An early possible duplication of *nifH* and paralogous comparisons make interpretations difficult (see Fig. 3 in Zehr et al., 2003). Four major clusters of *nifH* are recognized and functional nitrogenases are found in three of them (Zehr et al., 2003). The phylogenies of *nifH* genes are continuously revised and updated with novel sequences (including environmental ones) and more robust reconstruction methods. *nifH* genes from Gammaproteobacteria are found in different groups, as well as those from Betaproteobacteria (Bürgmann et al., 2004). Anomalies in the phylogenetic position of Betaproteobacteria have been reported as well (Hurek et al., 1997; Minerdi et al., 2001).

## Ecology of Dinitrogen-Fixing Prokaryotes

The communities of dinitrogen-fixing bacteria in natural environments may be studied with approaches such as the amplification by PCR of the nitrogenase reductase gene (*nifH*) with *nifH* primers using environmental DNA, with subsequent analyses by cloning and sequencing, by terminal restriction fragment length polymorphism (T-RFLP; Ohkuma et al., 1999; Tan et al., 2003), or by denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993). Hybridization to macro- and microarrays may reveal the presence and frequency of different $N_2$-fixing prokaryotes (Jenkins et al., 2004; Steward et al., 2004).

The ecology of the symbiotic $N_2$-fixing soil bacteria that are collectively designated rhizobia, has been comprehensively reviewed by Bottomley (1992), and ecogeographic and diversity reviews of these bacteria have been reported (Martínez-Romero and Caballero-Mellado, 1996; Sessitsch et al., 2002). Additional aspects of *Rhizobium* ecology in soil also have been reviewed (Sadowsky and Graham, 1998). *Frankia* symbiosis including some ecological aspects has been reviewed by Baker and Mullin (1992) and by Berry (1994). New molecular approaches have recently enhanced our perception of microorganisms in their natural habitats. By using PCR primers targeted to nitrogenase genes, the description and natural histories of communities of $N_2$-fixing microorganisms may be established more accurately than with traditional microbiological techniques. The fluctuations of marine diazotroph populations have been analyzed with these approaches. The bulk of $N_2$ fixation appears to shift from cyanobacterial diazotrophs in summer to bacterial diazotrophs in fall and winter (Zehr et al., 1995). The heterocystous cyanobacteria are not as efficient fixing nitrogen as the nonheterocystous cyanobacteria at the high temperatures of the tropical oceans (Staal, 2003). The diversity of marine $N_2$ fixers in benthic marine mats was determined from the sequences of *nifH* genes. The *nifH*

sequences obtained were most closely related to those of anaerobes, with a few related to Gammaproteobacteria including *Klebsiella* and *Azotobacter* species (Zehr et al., 1995).

The role of $N_2$ fixation was examined in microbial aggregates embedded in arid, nutrient-limited and permanent ice covers of a lake area in the Antarctic, and also in mats in soils adjacent to the ice border. Molecular characterization by PCR amplification of *nifH* fragments and nitrogenase activity measured by acetylene reduction showed a diverse and active diazotrophic community in all the sites of this environment. Nitrogenase activity was extremely low, compared to temperate and tropical systems. Diazotrophs may be involved in beneficial consortial relationships that may have advantages in this environment (Olson et al., 1998). Nitrogen fixation, observed in moderately decayed wood debris, was shown to be stimulated by warm temperatures (Hicks et al., 2003).

The diversity of the $N_2$-fixing microorganisms within the symbiotic community in the gut of various termites was studied without culturing the symbiotic microorganisms. Both small subunit (ss) rRNA (Kudo et al., 1998) and *nifH* genes (Ohkuma et al., 1999) were amplified in DNA extracted from the mixed microbial population of the termite gut. The analysis of the *nif* clones from diverse termites revealed different sequences in most of the individual termite species. Whereas the *nif* groups were similar within each termite family, they differed between termite families. Microorganisms from termites with high levels of $N_2$-fixation activity could be assigned to either the anaerobic *nif* group (clostridia and sulfur reducers) or to the alternative *nif* methanogen group. Highly divergent *nif* gene sequences (perhaps not even related to nitrogen fixation) were found in termites that showed low levels of acetylene reduction (Ohkuma et al., 1999). Expression of the $N_2$ fixation gene *nifH* was evaluated directly by amplifying *nifH* cDNA from mRNA by reverse transcription (RT)-PCR (Noda et al., 1999). Only the alternative nitrogenase (from *anf* gene) was preferentially transcribed in the gut of the termite *Neotermes koshunensis*. The levels of expression of the *anf* gene were related to the $N_2$ fixation activity recorded for the termites. The addition of Mo (molybdenum) to the termite diet did not repress the expression of the *anf* genes; however, Mo repression of other *anf* genes has been described (Noda et al., 1999). Estimates are that the contribution of insect-borne nitrogen-fixing bacteria in insects may be up to 30 kg of N/hectare (ha)/year (Nardi et al., 2002).

Endosymbionts from marine bivalve species, located in the shipworm gills, are cellulolytic and $N_2$-fixing. They provide cellulolytic enzymes to the host. They are a unique clade in the Gammaproteobacteria related to *Pseudomonas* and were designated as a new genus and species *Teredinibacter turnerae*, which fixes nitrogen in microaerobic in vitro conditions (Distel et al., 2002).

The arbuscular mycorrhizal fungus (*Gigaspora margarita*) has been shown to harbor a viable and homogeneous population of endosymbiotic bacteria that has been designated as "*Candidatus Glomeribacter gigasporarum*" (Bianciotto et al., 1996) related to Betaproteobacteria such as *Ralstonia* (Bianciotto et al., 2003). In the genomic library of total DNA from the fungal spores, clones carrying the bacterial genes *nifD* and *nifK* were identified. Both of these genes were arranged in a similar manner to the corresponding genes in archaea or bacteria and were similar to nitrogenases from different diazotrophs (Minerdi et al., 2001; Minerdi et al., 2002). mRNAs for the *nif* genes were detected, but whether these endosymbionts fix nitrogen is unknown.

Dinitrogen-fixing cyanobacteria form symbioses with diverse hosts such as fungi, bryophytes, cycads, mosses, ferns, and an angiosperm, *Gunnera* (Bergman et al., 1992). The genome of the cyanobacteria *Nostoc* (which is a symbiont of cycads, *Gunnera* and others) may be the largest among those from Prokaryotes, with nearly 10 Mb (Meeks et al., 2001).

New symbionts capable of forming nodules in the leguminous plant *Lotus corniculatus* were obtained in agricultural fields after the lateral transfer of genetic material to native nonsymbiotic soil mesorhizobia (Sullivan et al., 1995; Sullivan et al., 1996). Nonsymbiotic soil rhizobia, which outnumber symbiotic bacteria in some cases (Segovia et al., 1991; Laguerre et al., 1993), have been considered to be potential recipients of symbiotic plasmids. Molecular analyses (including the sequence of DNA fragments of 16S rRNA genes, the fingerprints of digested genomic DNA, and the hybridization patterns to cloned fragments) clearly demonstrated that a large segment of genetic material was acquired by soil *Mesorhizobium* bacteria (Sullivan et al., 1995) and that the original *Mesorhizobium loti* strain applied to the soil as an inoculant was the donor of these symbiotic genes. The mobilizable 500-kb DNA fragment has been designated a symbiosis island and it encodes genes for symbiotic $N_2$ fixation (*fix* genes) as well as those for the synthesis of vitamins (Sullivan et al., 2002). The symbiotic island was integrated into the phenylalanine-tRNA gene (Sullivan and Ronson, 1998). Interestingly, pathogenicity islands in other bacteria range up to 190 kb in size and most are either found adjacent to or integrated

within tRNA genes or flanked by insertion sequences (Cheetham and Katz, 1995; Kovach et al., 1996). In *M. loti*, the symbiotic genes are chromosomally located as in most *Mesorhizobium* and *Bradyrhizobium* sp. A similar symbiotic chromosomal region was identified in *M. loti* (Kaneko et al., 1999) that was later classified as *M. huakuii* (Turner et al., 2002). Only a few *Mesorhizobium* species such as *M. amorphae* possess symbiotic plasmids (Wang et al., 1999b), which are a common characteristic of *Rhizobium* and *Sinorhizobium* species (Martńez et al., 1990). The great chromosomal diversity, mainly based on 16S rRNA sequence (Wang and Martńez-Romero, 2000) and on glutamine synthetase (GSII) genes (Wernegreen and Riley, 1999) encountered in *M. loti*, may be ascribed to the natural occurrence of genetic transfer of symbiotic genes in *Mesorhizobium* (Sullivan et al., 1996).

The range of nodulating bacteria has enlarged. Nodulating *Methylobacterium* have been reported from *Crotalaria* nodules (Sy et al., 2001). Surprisingly, some Betaproteobacteria in the genera *Burkholderia* (Moulin et al., 2001) and *Ralstonia* (Chen et al., 2001) are capable of nodulating legumes. These bacteria have been classified as *Burkholderia phymatum*, *B. tuberum* (Vandamme et al., 2002), *B. caribensis* (Chen et al., 2003) and *Wautersia taiwanensis* (previously designated *Ralstonia taiwanensis*) (Chen et al., 2001; Vaneechoutte et al., 2004). Like *Rhizobium* and *Sinorhizobium* spp., these Betaproteobacteria possess symbiotic plasmids that carry nodulation genes (Chen et al., 2003). The similarity of these *nod* genes to those of the Alphaproteobacteria suggested that lateral transfer of *nod* genes occurred, most probably from Alpha- to Betaproteobacteria (Moulin et al., 2001; Chen et al., 2003). Similarly the lateral transfer of *nod* genes has been implied as a possible explanation for the nodulation capacity in *Devosia*, and a new species has been identified that carries *nodD* and *nifH* genes similar to those of *R. tropici* (Rivas et al., 2002).

## Dinitrogen-Fixing Prokaryotes in Agriculture

The first industrial production of *Rhizobium* inoculants began at the end of the nineteenth century. In the absence of nitrogen fertilization, spectacular increases in plant and seed yield may be obtained by inoculation of legumes where the specific rhizobia for the legumes are absent or scarce (Singleton and Tavares, 1986). Factors affecting nodule occupancy by rhizobia inoculants were reviewed by Vlassak and Vanderleyden (1997). Inoculation of soybean is a common practice in Brazil (Hungria et al., 2000) or in the United States where production of soybean inoculants is a top priority for inoculant-producing companies (Paau, 1989), and inoculation of cash crops with nitrogen-fixing inoculants is considered a realistic alternative to the ever increasing use of fertilizers. High quality inoculants (whose characteristics were discussed by Maier and Triplett, 1996) as well as the improvement of management systems, are useful not only for agriculture but also for reforestation of devastated areas. Leguminous trees with their corresponding rhizobia have been recommended for many and diverse uses including reforestation, soil restoration, lumber production, cattle forage, and for human food. The so-called "actinorhizal plants" that associate with *Frankia* are also of great value for reforestation; actinorhizal plants belong to eight families (Baker and Mullin, 1992; Berry, 1994).

A high impact goal of nitrogen fixation research has been to extend nitrogen fixation to non-legumes and this has promoted the search for nitrogen fixing bacteria that are associated with agriculturally valuable crops. From a basic research perspective this has increased our knowledge of their diversity. The impact on agriculture and potential as a substitute for the high levels of fertilizer used in intensive agriculture is debatable, and a critical review of the actual contributions of $N_2$ fixation to the amount of fixed N present in cereals and other grasses finds that $N_2$-fixing bacteria in agriculture provide only a limited amount of fixed N. Careful long-term N balance studies would be required to accurately estimate these contributions (Giller and Merckx, 2003). Levels of fixed nitrogen (as low as 5–35 kg N/ha per year) that contribute over the long term to sustain fertility in nonagricultural areas (Stevens et al., 2004) are neglible for present modern intensive agricultural needs but may be of use in traditional, low input small farming systems. Legumes may fix over 200 kg N/ha per year and this is a significant contribution of nitrogen. Conservative values for bacterial fixation in nonlegumes are 20–30 kg N/ha per year, but higher, substantial values have been also estimated (see below). The rate of fixation of the tree *Acacia dealbata* is considered sufficient to replace the estimated loss due to timber harvesting (May and Attiwill, 2003).

Sugarcane and rice are the Gramineae most extensively studied with regard to $N_2$ fixation, but other crops are being studied as well (see below). Sugarcane has been grown for more than 100 years in some areas of Brazil without nitrogen fertilization or with very low nitrogen inputs, and removal of the total harvest has not led to decline in yield and soil nitrogen levels. This observation suggested that $N_2$ fixation may have been the source for a substantial part of the

nitrogen used by this crop (Dɒbereiner, 1961). Alternatively, irrigation water has been implicated as a possible source of N (Giller and Merckx, 2003).

From 25–55% (Urquiaga et al., 1989; Yoneyama et al., 1997) or perhaps as much as 60–80% (Boddey et al., 1991) of the plant N could be derived from associative dinitrogen fixation, but scepticism about the occurrence of high levels of nitrogen fixation has been expressed (Giller and Merckx, 2003). The problems of estimating sugarcane $N_2$ fixation, discussed by Boddey et al. (1995), include different patterns of nitrogen uptake by different sugarcane varieties (Urquiaga et al., 1989), declining $^{15}N$ enrichment of soil mineral nitrogen, carryovers of nitrogen from one harvest to the next, and differential effects on control plants during the three-year study (Urquiaga et al., 1992). The mean estimates of fixed $N_2$ for two sugarcane hybrids grown in concrete tanks ranged from 170–210 kg $N_2$ fixed/ha (Urquiaga et al., 1992). Correction for micronutrient soil deficiencies and high soil moisture seem to be key conditions that promote $N_2$ fixation in sugarcane plants (Urquiaga et al., 1992). The evidence of large differences in $N_2$ fixation among different sugarcane cultivars is compelling.

Dinitrogen-fixing bacteria isolated from the rhizosphere, roots, stems and leaves of sugarcane plants include *Beijerinckia*, *Azospirillum*, *Azotobacter*, *Erwinia*, *Derxia*, *Enterobacter* (reviewed in Boddey et al., 1995), *Gluconacetobacter* (Cavalcante and Dɒbereiner, 1988), and *Herbaspirillum* (Baldani et al., 1986). *Gluconacetobacter diazotrophicus* has the capacity to fix $N_2$ at low pH and in the presence of nitrate and oxygen. A *G. diazotrophicus nifD* mutant that cannot fix $N_2$ has been tested on plants derived from tissue cultures. Plant height was significantly increased by the wildtype strain and not by the mutant strain inoculants, suggesting a positive effect of $N_2$ fixation by *G. diazotrophicus* on sugarcane (Sevilla et al., 1998). Beneficial effects of *G. diazotrophicus* inoculation in experimental fields also have been reported (Sevilla et al., 1999), but global N balances were not analyzed. Selected strains of *Herbaspirillum* were reported to stimulate plant development (Baldani et al., 1999). *Gluconacetobacter diazotrophicus* (James and Olivares, 1997), *Herbaspirillum seropedicae* and *H. rubrisubalbicans* (Olivares et al., 1996) have been clearly shown to colonize sugarcane plants internally. Colonization by *G. diazotrophicus* was inhibited by nitrogen fertilization (Fuentes-Ramírez et al., 1999). Probably $N_2$ fixation in sugarcane is performed by a bacterial consortium.

Several studies have been carried out on nitrogen balance in lowland rice fields in Thailand (Firth et al., 1973; Walcott et al., 1977), in Japan (Koyama and App, 1979), and at the experimental fields of the International Rice Research Institute (IRRI) in the Philippines (App et al., 1984; Ventura et al., 1986). These studies report a positive balance with estimates of around 16–60 kg of nitrogen fixed per ha per crop (App et al., 1986; Ladha et al., 1993). In a nitrogen-balance study carried out on 83 wild and cultivated rice cultivars (6 separate experiments, each with 3 consecutive crops), large and significant differences between cultivars were found (App et al., 1986). But other assays showed only a small or nonsignificant contribution of fixed $N_2$ in rice (Watanabe et al., 1987b; Boddey et al., 1995).

Many different $N_2$-fixing bacteria have been isolated from rice roots. These include *Azotobacter*, *Beijerinckia* (Dɒbereiner, 1961), *Azospirillum* (Baldani and Dɒbereiner, 1980; Ladha et al., 1982), *Pseudomonas* (Qui et al., 1981; Barraquio et al., 1982; Barraquio et al., 1983; Watanabe et al., 1987a; Vermeiren et al., 1999), *Klebsiella*, *Enterobacter* (Bally et al., 1983; Ladha et al., 1983), *Sphingomonas* (described as *Flavobacterium* in Bally et al., 1983), *Agromonas* (Ohta and Hattori, 1983), *Herbaspirillum* spp. (Baldani et al., 1986; Olivares et al., 1996), sulfur-reducing bacteria (Durbin and Watanabe, 1980; reviewed in Barraquio et al. [1997] and in Rao et al. [1998]), *Azoarcus* (Engelhard et al., 1999) and methanogens (Rajagopal et al., 1988; Lobo and Zinder, 1992). The nitrogenase genes of *Azoarcus* are expressed on rice roots (Egener et al., 1998), and *Herbaspirillum seropedicae* expresses *nif* genes in several gramineous plants including rice (Roncato-Maccari et al., 2003).

Cyanobacteria have long been used to fertilize agricultural land throughout the world, most notably rice paddies in Asia. Increases in rice plant growth and increases in nitrogen content in the presence of cyanobacteria have been documented by many investigators. Plant promotion may also be related to growth-promoting substances produced by the cyanobacteria (Stewart, 1974). *Azolla* is a small freshwater fern that grows very rapidly on the surface of lakes and canals. Extensive employment of *Azolla-Anabaena* as a green manure in rice cultivation has been documented. *Anabaena*, a representative filamentous cyanobacterium, establishes symbioses with a diversity of organisms including *Azolla*. Unfortunately, various cyanobacteria also produce highly poisonous toxins and some of them are related to the high incidence of human liver cancer in certain parts of China. Highly toxic strains have been found in *Anabaena* and in other genera of cyanobacteria, and identification of such strains requires sophisticated biochemical tests (Carmichael, 1994).

Alternatively, other bacterial species are being tested to promote rice growth, such as the $N_2$-fixing *Burkholderia vietnamiensis* (Gillis et al., 1995). In some agriculture sites in Vietnam, this species has been isolated as the dominant $N_2$-fixing bacterium in the rice rhizosphere (Trân Van et al., 1996). *Burkholderia vietnamiensis* inoculation has resulted in significant increases (up to 20%) in both shoot and root weights in pots and its use in rice inoculation seems highly promising (Trân Van et al., 1994). However, a note of caution has been raised with a proposed moratorium on the agricultural use of *B. vietnamiensis*, which has a close genetic relationship to human pathogens implicated in lethally infecting patients with cystic fibrosis (Holmes et al., 1998). Detailed molecular analysis may allow for the distinction of pathogenic and environmental isolates (Segonds et al., 1999).

For over seven centuries, rice rotation with clover has significantly benefited rice production in Egypt. Clover is normally associated with *Rhizobium leguminosarum* bv. *trifolii* that forms $N_2$-fixing nodules in the root of this plant. Surprisingly, strains of this bacterium also were encountered inside the rice plant with around $10^4$–$10^6$ rhizobia per gram (fresh weight) of root. These values are within the range of other bona fide endophytic bacteria (Yanni et al., 1997). Promotion of rice shoot and root growth was dependent on the rice cultivar, inoculant strain, and other conditions. Inoculation of rice with a selected strain gives best results in presence of low doses of nitrogen fertilizer. A number of investigators have reported growth stimulation of crops such as wheat and corn inoculated with a *R. leguminosarum* bv. *trifolii* strain, but these effects may not be related to $N_2$ fixation (Holflich et al., 1995).

In non-legumes (such as *Arabidopsis thaliana* [a model plant]), penetration of rhizobial strains has been found to be independent of nodulation genes that are normally required for bacterial entry into the legume root (Gough et al., 1996; Gough et al., 1997; Webster et al., 1998; O'Callaghan et al., 1999). This process probably requires cellulases and pectinases (Sabry et al., 1997). *Azorhizobium caulinodans*, in addition to forming nodules on *Sesbania rostrata*, has been found to colonize the xylem of its host (O'Callaghan et al., 1999) as well as to colonize wheat (Sabry et al., 1997). In wheat, *A. caulinodans* promotes increases in dry weight and nitrogen content as compared to uninoculated controls; acetylene reduction activity was also recorded. The interaction between azorhizobia and wheat root resembles the invasion of xylem vessels of sugarcane roots by *G. diazotrophicus* (James and Olivares, 1997) and *Herbaspirillum* spp. (Roncato-Maccari et al., 2003) and of wheat by *Pantoea agglomerans* (Ruppel et al., 1992). The xylem vessels may be the site of $N_2$ fixation because they provide the necessary conditions (carbohydrates and low oxygen tension), although the nutrient levels in the xylem have been considered as too low to maintain bacterial growth and $N_2$ fixation (Fuentes-Ramírez et al., 1999; Welbaum et al., 1992). In acreage cultivated using *Sesbania rostrata*-rice rotation, *A. caulinodans* survives in the soils and rhizosphere of wetland rice (Ladha et al., 1992). *Azorhizobium caulinodans* can colonize the rice rhizosphere (specifically around the site of lateral root emergence), penetrate the root at the site of emergence of lateral roots, and colonize subepidermally intercellular spaces and dead host cells of the outer rice root cortex (Reddy et al., 1997).

The application of green manure has been an agronomic practice for increasing rice production, and legumes also can be used because of their symbiosis with $N_2$-fixing rhizobia. A large number of species are used both before and after rice culture including *Macroptilium atropurpureum*, *Sesbania* and *Aeschynomene* spp. (Ladha et al., 1992). Owing to their high $N_2$-fixing capacity and their worldwide distribution, flood-tolerant legumes such as *Sesbania rostrata* have been the focus of research. *Sesbania herbacea* nodulated by *R. huautlense* is also a flood-tolerant symbiosis (Wang and Martínez-Romero, 2000).

Nitrogen fixation in non-legumes is conditioned more by the plant than by the bacteria. Interestingly, aluminum-tolerant plants are more capable of maintaining bacterial nitrogen fixation than plants that are not tolerant (Christiansen-Weniger et al., 1992), maybe because they excrete dicarboxylics that are adequate to support bacterial $N_2$-fixation.

$N_2$-fixing bacteria associated to maize include: *Azospirillum*, *Herbaspirillum*, *Klebsiella* (Chelius and Triplett, 2001), *Burkholderia vietnamiensis* (Trân Van et al., 1996), *R. etli* (Gutiérrez-Zamora and Martínez-Romero, 2001), and the newly described species (*Paenibacillus brasilensis*; [Von der Weid et al., 2002] and *Klebsiella variicola* [Rosenblueth et al., 2004]). *Klebsiella variicola* was also found associated with banana plants (Martínez et al., 2003). Soil type instead of the maize cultivar determined the structure of a *Paenibacillus* community in the rhizosphere (Araujo de Silva et al., 2003).

Sweet potato (*Ipomoea batatas*) may grow in poor N-soil and associated N-fixation has been considered to contribute N to these plants. By a cultivation-independent approach, bacteria similar to *Klebsiella*, *Rhizobium* and *Sinorhizobium* were inferred to be present as sweet potato endophytes (Reiter et al., 2003).

## Perspectives of Application of Nitrogen Fixation Research

The transgenic plants that will herald a revolution in agriculture are those with functional nitrogenase genes that, when expressed, will satisfy all the plant's nitrogen needs. The source of these genes will be prokaryotic. Research efforts are directed towards the ambitious goal of transforming rice plastids (Potrykus group in Zürich discussed in Rolfe et al., 1998) and plastids of the alga *Chlamydomonas reinhardtii* (Dixon et al., 1997; Dixon, 1999). Introduction of additional genes into plants to protect nitrogenase from oxygen damage will be needed. Such approaches could only be based on a profound understanding of $N_2$ fixation biochemistry, gene regulation and organization, as well as the structure and function of nitrogenases. Whether such a goal is feasible is difficult to predict.

The identification and selection of plant-associated microorganisms and their genetic improvement is an alternative strategy for obtaining agricultural crops that benefit from prokaryotic $N_2$ fixation. $N_2$ fixation ($N_2$ fixation without nodules) from associated bacteria is being considered as a suitable mode to exploit $N_2$ fixation in non-legumes (Triplett, 1996). Rhizosphere $N_2$ fixation by *Rahnella aquatilis* has been reported to occur in maize and wheat (Berge et al., 1991), and in other plants (Heulin et al., 1989). Mycorrhiza associate with most plants, and interestingly, bacteria-like organisms with nitrogenase genes have been found to be natural endosymbionts of the mycorrhiza (Minerdi et al., 2002). This association may be exploited to transfer $N_2$ fixation to non-legumes. The genetic improvement of mycorrhiza and bacterial symbionts may constitute a highly efficient system for the provision of fixed nitrogen to the plants.

The usefulness of $N_2$-fixing bacteria in bioremediation is also being recognized (Suominen et al., 2000; Prantera et al., 2002). Increased transformation of contaminating polychlorinated biphenyls was obtained with alfalfa inoculated with *Sinorhizobium meliloti* at 44 days after planting (Mehmannavaz et al., 2002). Dinitrogen fixation may decrease the need for nitrogen required by bacterial consortia used to degrade diesel fuel (Piehler et al., 1999).

Novel $N_2$ fixers may be found if the enrichment conditions for their isolation are more varied so as to include aerobic, anaerobic or microaerobic conditions, a variety of carbon sources at varying concentrations (copiotrophic and oligotrophic conditions; Kuznetsov et al., 1979), and media formulations that include or exclude Mo or V. The discovery of a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase from *Streptomyces thermoautotrophicus* (Ribbe et al., 1997) opens a new avenue in $N_2$ fixation research. Undoubtedly, other microorganisms containing this nitrogenase have yet to be identified. This nitrogenase may prove to be more amenable for introduction into plants because of its lower energy requirements and its higher tolerance to oxygen.

Elevated $CO_2$ levels provided to legumes were found to stimulate $N_2$ fixation indicating that $N_2$ fixation was limited by the availability of photosynthate (Zanetti et al., 1996). Environmental and management constraints to legume growth (basic agronomy, nutrition, water supply, diseases, and pests) are the major limiting factors of $N_2$ fixation in many parts of the world. Crop production on 33% of the world's arable land is limited by phosphorus availability (Sánchez and Vehara, 1980). Efforts to maximize the input of biologically fixed nitrogen into agriculture will require concurrent approaches, which include the alleviation of phosphorus and water limitation, the enhancement of photosynthate availability, as well as sound agricultural management practices.

## Biochemistry and Physiology of Dinitrogen Fixation

Although the chemical nature of the primary product of $N_2$ fixation was the subject of debate for many years, the issue was clarified with the use of $^{15}N$. All diazotrophs were thought to use the same two-component nitrogenases (consisting of an iron and an molybdenum-iron protein). Alternative nitrogenases were reported subsequently (Hales et al., 1986; Robson et al., 1986) and found in very different bacteria including *Anabaena variabilis*, *Azospirillum brasilense*, *Clostridium pasteurianum*, *Heliobacter gestii*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and bacteria corresponding to Gammaproteobacteria such as *Pseudomonas* (Saah and Bishop, 1999). *Azotobacter vinelandii*, an aerobic soil bacterium, was the first diazotroph shown to have three distinct nitrogenases: the classical molybdenum (Mo)-containing nitrogenase (nitrogenase 1), the vanadium (V)-containing (nitrogenase 2), and the iron-only nitrogenase (nitrogenase 3; Maynard et al., 1994). The alternative nitrogenases (nitrogenase 2) use V instead of Mo, and this substitution is advantageous under conditions where Mo is limiting (Jacobitz and Bishop, 1992). Similarly, the iron nitrogenase (nitrogenase 3) is expressed only in Mo- and V-deficient, nitrogen-free media. The V-containing nitrogenase produces around three times more hydrogen than the Mo-nitrogenase (Eady, 1996).

A Mo-dinitrogenase and a manganese-superoxide oxidoreductase have been found to couple $N_2$ reduction to the oxidation of superoxide. This nitrogenase is more efficient than the classical enzyme, which requires a fourfold greater input of ATP. This $N_2$-fixing system, which is not sensitive to oxygen, has only been described in *Streptomyces thermoautotrophicus* (Ribbe et al., 1997), and the genomic DNA of this bacterium does not hybridize to DNA probes for the classical *nif* genes. Although the overall reactions catalyzed by *S. thermoautotrophicus* are similar to those of previously characterized nitrogenases (e.g., the production of $H_2$), it is the subunit structure, polypeptides, and inability to reduce acetylene that distinguishes the nitrogenase of this system from other nitrogenases (Ribbe et al., 1997). The currently known dinitrogenase reductases are ca. 63-kDa $\gamma_2$ dimeric iron proteins that contain 4 Fe and 4 $S^{-2}$ per dimer. In contrast, the St2 protein of *S. thermoautotrophicus* has been identified as a member of the manganese-superoxide oxidoreductases (SODs) with molecular mass ~48 kDa and no Fe or $S^{-2}$. Unlike other SODs, St2 cannot convert $O^-_2$ into $O_2$ and $H_2O_2$. Some diazotrophs are able to utilize the $H_2$ evolved from $N_2$ fixation via uptake hydrogenases (Evans et al., 1985). These enzymes are found in $N_2$-fixing and non-$N_2$-fixing bacteria and in cyanobacteria. The uptake hydrogenases in *Anabaena* are present only in heterocysts, which are the specialized $N_2$-fixing cells of cyanobacteria; interestingly, the hydrogenase genes are rearranged during heterocyst differentiation (Carrasco et al., 1995).

Hitherto, ammonium has been accepted as the primary product of $N_2$ fixation and as a reactant in the biosynthesis of all nitrogen-containing molecules made by $N_2$-fixing organisms. Because ammonia excretion has been considered a beneficial characteristic enabling $N_2$ fixers to establish symbioses with other organisms such as plants, it has been generally assumed that the ammonium assimilation enzymes are depressed in symbiotic bacteria. However, *Bradyrhizobium japonicum*, which forms nodules and fixes nitrogen in soybean plants has been shown to excrete alanine preferentially and not ammonium (Waters et al., 1998). Whether this generally occurs in rhizobia is still controversial (Youzhong et al., 2002; Lodwig et al., 2003; Lodwig et al., 2004). The ratio of alanine to ammonia excretion seems to be related to the oxygen concentration and the rate of respiration (Li et al., 1999). For the cyanobacterium *Nostoc*, which can establish symbiosis with many organisms including *Gunnera*, ammonia excretion accounts for only 40% of the nitrogen released (Peters and Meeks, 1989). Different plant endophytes have been found to release (excrete) riboflavin during $N_2$ fixation (Phillips et al., 1999b). Lumichrome, a compound obtained from riboflavin, has been reported to stimulate root respiration and promote alfalfa seedling growth (Phillips et al., 1999a). Production of riboflavin-lumichrome by plant-associated bacteria is favored by a high N-to-C ratio in the media, and possibly $N_2$ fixation also promotes the synthesis of nitrogen-containing compounds (other than ammonia), such as lumichrome, that can benefit plants.

Nitrogenase Structure The classical nitrogenase is a complex, two-component metalloprotein composed of an iron (Fe) protein and a molybdenum-iron (MoFe) protein. The properties of nitrogenase have been reviewed (Howard and Rees, 1994; Burgess and Lowe, 1996; Eady, 1996; Seefeldt and Dean, 1997). The iron-molybdenum cofactor (Fe-Moco), the prototype of a small family of cofactors, is a unique prosthetic group that contains Mo, Fe, S, and homocitrate in a ratio of 1 : 7 : 9 : 1, and it is the active site of substrate reduction (Hoover et al., 1989; Kim and Rees, 1992b). All substrate reduction reactions catalyzed by nitrogenase require the sequential association and dissociation of the two nitrogenase components.

A great deal of effort to define the structure of nitrogenases has been expended. *Azotobacter vinelandii* has been suitable for these studies because it produces large amounts of the enzyme, it is amenable to genetic manipulation, and it has *nif* and *nif*-associated genes of known sequence (Brigle et al., 1985; Jacobson et al., 1989; Bishop and Premakumar, 1992). A major achievement in the biochemistry of nitrogenases has been the establishment of the structure of the Fe (Georgiadis et al., 1992) and the MoFe proteins (Kim and Rees, 1992b; Bolin et al., 1993; Schindelin et al., 1997) involving high resolution X-ray crystallographic analysis (Peters et al., 1997; Schlessman et al., 1998). A ~2.2 Å resolution has been reported for the *Azotobacter vinelandii* MoFe-protein (Peters et al., 1997), the *A. vinelandii* Fe-protein (Av2), and the *Clostridium pasteurianum* Fe-protein (Schlessman et al., 1998). The knowledge of the Fe protein structure has contributed to understanding how MgATP functions in nitrogenase catalysis. The Fe-protein is a homodimer with two ATP-binding sites, and the nucleotide binding causes conformational changes in the protein. ATP hydrolysis occurs in the transient complex formed between the component proteins. Molecular interactions were proposed from mutagenesis studies of the nitrogenases (Kent et al., 1989; Dean et al., 1990; Scott et al., 1990). Site-specific mutagenesis studies based on the FeMo protein crystal structure (Kim and Rees, 1992a) have been aimed at amino acids related to the FeMo-cofactor (espe-

cially at the residues proposed to be involved in the entry and exit path for substrates, inhibitors and products) and also at those residues involved in FeMo-cofactor insertion during biosynthesis. The spectroscopic and kinetic properties of the resulting mutant proteins are studied (Dilworth et al., 1998).

The use of biophysical, biochemical and genetic approaches have facilitated the analysis of the assembly and catalytic mechanisms of nitrogenases. The synthesis of the prosthetic groups of nitrogenases has been a challenge for chemists. The different substrates utilized by the nitrogenases seem to bind to different areas of the FeMo-cofactor (Shen et al., 1997). Nitrogenase structural changes that occur after the formation of the active complex are thought to produce transient cavities within the FeMo protein, which when opened allows the active site to become accessible (Fisher et al., 1998). The FeMo-cofactor also is found associated with the alternative nitrogenase, *anf*-encoded proteins (AnfDGK; Gollan et al., 1993; Pau et al., 1993).

The *nifDK* genes of *Azotobacter vinelandii* were fused and then translated into a single large nitrogenase protein that interestingly has nitrogen fixation activity (Suh et al., 2003). This shows that the MoFe protein is flexible. However a substitution of tungsten for Mo abolished nitrogenase activity (Siemann et al., 2003).

*Nitrogen Fixation Genes* The complete nucleotide sequence of the *Klebsiella pneumoniae* 24-kb region required for $N_2$ fixation was reported in 1988 (Arnold et al., 1988). Genes for transcriptional regulators were found to cluster contiguously with the structural genes for the nitrogenase components and genes for their assembly. The $N_2$ fixation (*nif*) genes are organized in seven or eight operons containing the following *nif* genes: *J*, *H*, *D*, *K*, *T*, *Y*, *E*, *N*, *X*, *U*, *S*, *V*, *W*, *Z*, *M*, *F*, *L*, *A*, *B* and *Q* (Fig. 2). The products of at least six $N_2$ fixation (*nif*) genes are required for the synthesis of the iron-molybdenum cofactor (FeMo-co): *nifH*, *nifB*, *nifE*, *nifN*, *nifQ*, and *nifV*. NifU and NifS might have complementary functions mobilizing the Fe and S respectively needed for nitrogenase metallocluster assembly in *A. vinelandii*. Notably, some of the gene products required for forma-

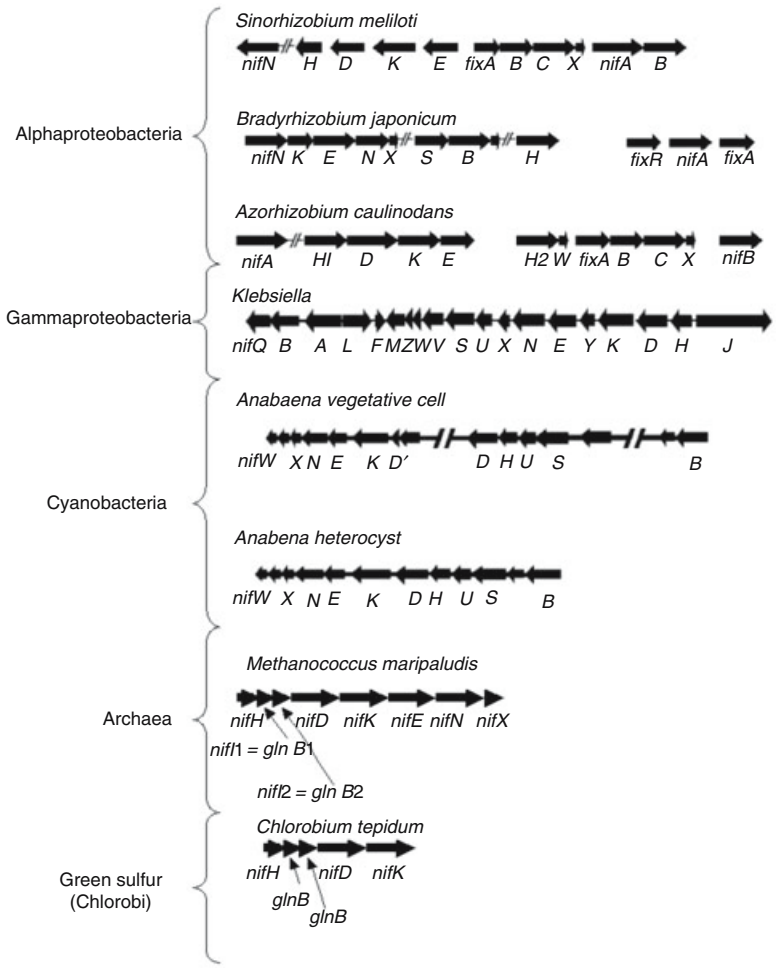


Fig. 2. Arrangements of *nif* genes in dinitrogen-fixing prokaryotes. The *nif* gene organization in *Methanococcus maripaludis* is from Kessler et al. (2001).

tion of the Mo-dependent enzyme are also required for maturation of alternative nitrogenases (Kennedy and Dean, 1992). The *nifJ* gene of *Klebsiella* is required for $N_2$ fixation, but in the cyanobacterium *Anabaena*, NifJ is required for $N_2$ fixation only when Fe is limiting (Bauer et al., 1993), whereas in *R. rubrum*, a NifJ protein does not seem to be required for $N_2$ fixation (Lindblad et al., 1993). The organization of *nif* genes in *Anabaena* is unique and different from that of other $N_2$ fixers because *nifD* is split between two DNA fragments separated by 11 kb. Recombination events are required to rearrange a contiguous *nifD* gene in $N_2$-fixing cells (Haselkorn and Buikema, 1992; Fig. 2).

A detailed analysis of the gene products of *nifDK* and *nifEN* (Brigle et al., 1987) revealed a possible evolutionary history involving two successive duplication events. A duplication of an ancestral gene that encoded a primitive enzyme with a low substrate specificity might have occurred before the last common ancestor of all living organisms emerged (Fani et al., 1999).

Nitrogenase structural genes are located on plasmids in some bacteria (such as *Rahnella aquatilis* [Berge et al., 1991], *Enterobacter*, and *Rhizobium* spp. [Martínez et al., 1990]) but are chromosomally encoded in the majority of prokaryotes including bradyrhizobia and most mesorhizobia.

The repeated sequences clustered around the *nif* region of the *Bradyrhizobium japonicum* genome may be involved in recombination thereby facilitating the formation of deletions (Kaluza et al., 1985). In *R. etli* bv. *phaseoli*, multiple copies of the *nif* operon promote major rearrangements in the symbiotic plasmid at high frequency (Romero and Palacios, 1997). Differences in the promoter sequences of the *nifH* regions in *R. etli* are correlated with the different levels of *nif* gene expression (Valderrama et al., 1996). The symbiotic plasmid of *R. etli* bv. *mimosae* is closely related to that of bv. *phaseoli* but its *nif* gene has a different restriction fragment length polymorphism (RFLP) pattern as revealed by *nifH* gene hybridization (Wang et al., 1999a).

A conserved short nucleotide sequence upstream of genes regulated by oxygen (i.e., an anaerobox) has been detected upstream of *Azorhizobium caulinodans nifA* (Nees et al., 1988), *Bradyrhizobium japonicum hemA*, *S. meliloti fixL*, *fixN*, *fixG*, in front of an open reading frame located downstream of *S. meliloti fixS*, within the coding region of *R. leguminosarum* bv. *viciae fixC*, i.e., upstream of the *nifA* gene and upstream of the *fnr* gene (*fixK*-like).

Alternative nitrogenase genes, *anfH*, *anfD* and *anfG* (Mo-independent) are found in the termite gut diazotrophs. The sequences of these genes are similar to those found in bacteria even though the gene organization with contiguous GlnB-like proteins resembles that found in the Archaea (Noda et al., 1999).

The existence of structural genes for three different nitrogenases was revealed when the complete genome sequence of the photosynthetic bacterium *Rhodopseudomonas palustris* was determined (Larimer et al., 2004). Previously, only *Azotobacter* sp. was known to possess three nitrogenases. The expression of *nif* genes of *Azotobacter vinelandii* was determined directly in soil by PCR amplification of reverse transcribed *nifH* gene fragments using *nifH* primers specific for *A. vinelandii* (Bürgmann et al., 2003).

*Regulation of Nitrogen Fixation Genes* Since nitrogen fixation is an energy expensive process, it is finely tuned, with transcriptional as well as posttranslational regulation. *nif* genes are normally not expressed and require transcriptional activation when N is limiting and conditions are appropriate for nitrogenase functioning. If little is known about the extant diazotrophs, less is known about $N_2$ fixation gene regulation from a global phylogenetic perspective. Most studies have been directed to Proteobacteria. For actinobacteria and firmicutes there is almost no information. Cyanobacteria and more recently Archaea were studied and showed very different regulation mechanisms from the ones observed in Proteobacteria. In Archaea, a repressor of *nif* genes has been identified (Lie and Leigh, 2003) and no *nifA* has been found in cyanobacteria (Herrero et al., 2001).

Novel regulatory elements, their fine interaction, and a huge complexity of regulatory networks are being revealed as the regulation of nitrogen fixation is studied in depth in model bacterial species. The results are revealing a very complicated sequence of regulatory cascades (Dixon, 1998; Nordlund, 2000; Forchhammer, 2003; Zhang et al., 2003). Regulatory elements such as $P_{II}$ (also known as *glnB*), DRAT (that transfers a ribosyl to nitrogenase and interferes with its activity), and DRAG (that removes the ribosyl) have been found in many diverse nitrogen fixing or non-nitrogen fixing Proteobacteria, Actinobacteria and Archaea (Ludden, 1994; Zhang et al., 2003). Very diverse modes of regulation of *nif* genes have been described that vary between species or even between strains in a single species (D'hooghe et al., 1995; Girard et al., 2000). Detailed studies have been carried out in *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Azospirillum brasilense*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, etc. The most common nitrogenases studied are inactivated by oxygen, and accordingly, the expression

of *nif* genes is negatively regulated by high oxygen concentrations. Different oxygen protection mechanisms have been described (reviewed by Vance, 1998).

Some of the bacterial diazotrophs share a common mechanism of transcriptional initiation of *nif* genes using a RNA polymerase holoenzyme containing the alternative sigma factor $\sigma^N$ ($\sigma^{54}$) and the transcriptional activator NifA (Kustu et al., 1989). Regulators of NifA vary among different diazotrophs. Factor $\sigma^N$ is competent to bind DNA, but the formation of the open promoter complex (active for transcriptional initiation) is catalyzed by NifA in a reaction requiring nucleoside triphosphate hydrolysis (Lee et al., 1993; Austin et al., 1994). The dual regulation by $\sigma^{54}$ and NifA may be required to ensure a stringent regulation of *nif* gene expression, and this may be so because biological $N_2$ fixation represents a major energy drain for the cell. In addition it seems reasonable that *nif* genes are negatively regulated by ammonia to avoid production of the enzyme in the presence of available fixed nitrogen; accordingly, nitrogenase enzymes are inactivated by ammonia but to a lesser degree in *Gluconacetobacter diazotrophicus* (Perlova et al., 2003).

In vivo DNA protection analysis demonstrated that NifA binds to the upstream activator sequences of *nif* genes (Morett and Buck, 1988). In the Alpha- and Betaproteobacteria, the activity of NifA is modulated negatively by the anti-activator NifL, which is a flavoprotein. The integrated responses to fixed nitrogen, oxygen, and energy status are mediated via NifL. The oxidized form of NifL inhibits NifA activity. A potential candidate Fe-containing electron donor involved in the signal transduction of NifL may be a flavohemoglobin, which may act as a global intracellular oxygen sensor (Poole et al., 1994). The expression of *nifL* and *nifA* in *Klebsiella pneumoniae* are coupled at the translational level (Govantes et al., 1998). Mutant forms of NifA were obtained that are no longer inhibited by NifL in *Azotobacter vinelandii* (Reyes-Ramírez, 2002).

In other diazotrophic Proteobacteria, the NifA protein itself senses oxygen probably via a cysteine-rich motif between the central domain and the C-terminal DNA-binding domain (Fischer et al., 1988). Oxygen-tolerant variants of the *S. meliloti* NifA proteins have been obtained (Krey et al., 1992). Ammonium-insensitive NifA mutants have been reported with modifications involved in the N-terminus of the molecule in *Herbaspirillum seropedicae*, *Azospirillum brasilense* and *Rhodobacter capsulatus* (Souza et al., 1995; Arsene et al., 1996; Kern et al., 1998).

In *Klebsiella pneumoniae*, the *nif* mRNAs were found to be very stable under conditions favorable to $N_2$ fixation, but the half lives of the *nifHDKTY* were reduced several fold when adding $O_2$ or fixed nitrogen. A fragment of the *nifH* sequence is required for the $O_2$-regulation of mRNA stability, and NifY may be involved in the sensing process (Simon et al., 1999).

Symbiotic nitrogen fixation shares common elements with free-living nitrogen fixation, but there are substantial differences as well. In *Rhizobium*, $N_2$ fixation only takes place inside the nodule. Still not well understood is how the plant partner influences the $N_2$-fixing activity of the microsymbiont, and the same is true for termite-diazotroph symbioses as well as for cyanobacteria in plants. In the latter case, the plant seems to stimulate the formation of heterocysts, which are differentiated cells that fix $N_2$ (Wolk, 1996). Even among symbiotic bacteria of legumes (*Sinorhizobium*, *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*), differences in the fine mechanisms regulating $N_2$ fixation exist and have been reviewed (Fischer, 1994; Kaminski et al., 1998). In *S. meliloti*, *fixLJ* (David et al., 1988) gene products belong to a two-component regulatory family of proteins that are responsive to oxygen. FixL is a high affinity oxygen sensor hemoprotein that has kinase-phosphate activity and is involved in phosphorylation of FixJ in microoxic or anoxic conditions (Gilles-Gonzalez et al., 1994). Upon phosphorylation, FixJ binds to the *nifA* and *fixK* promoters and allows their transcriptional activation (Waelkens et al., 1992).

Nitrogen fixation takes place in heterocysts in some cyanobacteria. Heterocyst differentiation is regulated by HetR, a protease (Haselkorn et al., 1999), and is inhibited by ammonia (Wolk, 1996). The expression of *nif* genes is also down-regulated by ammonium or nitrate (Thiel et al., 1995; Muro-Pastor et al., 1999). NtcA is a regulator required for expression of ammonium-repressible genes; in a *ntcA* mutant, induction of *nifHDK* and *hetR* is abolished or minimal (Frias et al., 1994; Wei et al., 1994). The *ntcA* gene, which is conserved among cyanobacteria, bears a DNA-binding motif close to the C-terminus and is homologous to *E. coli* Crp and to *S. meliloti* FixK. The NtcA protein binds to defined sequence signatures that are located upstream of ammonium-regulated promoters (Luque et al., 1994). However, no such signature has been identified upstream of *nif* or *hetR* genes. The *ntcA* gene is autoregulated and presumed activators or cofactors may render NtcA active (Muro-Pastor et al., 1999).

Biological $N_2$ fixation requires a minimum of 16 ATP molecules and 8 reducing equivalents per molecule of $N_2$ reduced. Under physiological conditions, a small electron carrier such as a ferredoxin or a flavodoxin is thought to transfer electrons to nitrogenase. In the photosynthetic

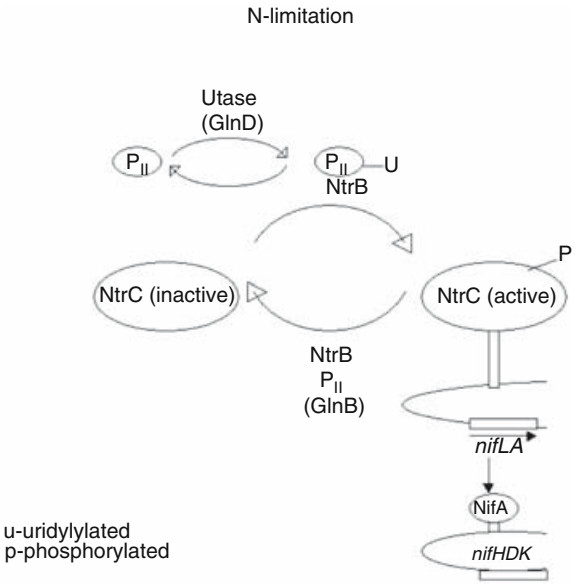


Fig. 3. Cascade regulatory mechanisms in the γ and β *Proteobacteria* under N-limited conditions. Uridylylated $P_{II}$, as a cofactor of NtrB promotes the phosphorylation of NtrC that then becomes active to bind the upstream regulatory sequences (UAS) of the *nifLA* promoter. NifA in turn binds the UAS of the nitrogenase structural genes in many dinitrogen-fixing prokaryotes studied, allowing their expression and consequentely nitrogen fixation.

bacterium *Rhodobacter capsulatus*, a ferredoxin Fd1 was identified as the major electron donor to nitrogenase (Schatt et al., 1989; Schmehl et al., 1993).

Conclusions Dinitrogen fixation is an important biological process carried out only by prokaryotes. Research on nitrogen fixation has followed a multidisciplinary approach that ranges from studies at the molecular level to practical agricultural applications. Support for research in this area has been driven by economic and environmental imperatives on the problems associated with the use of chemically synthesized nitrogen fertilizer in agriculture (Brewin and Legocki, 1996; Vance, 1998). However, the contributions of researchers in $N_2$ fixation to gene regulation, biochemistry, physiology, microbial ecology, protein assembly, and structure, and more recently to genomics are highly meritorious achievements in themselves.

Dinitrogen fixation research is a fast evolving field with specific model systems studied in great depth and an extensive knowledge of a larger diversity of $N_2$-fixing prokaryotes more slowly developing. The advent of molecular biology has certainly enriched our knowledge of the reservoir of $N_2$-fixing microorganisms and their ecology, but still the estimates of the amounts of nitrogen fixed in nature are uncertain. Human activities are liberating huge amounts of fixed nitrogen to the environment (Socolow, 1999; Karl et al., 2002; McIsaac et al., 2002; Van Breemen et al., 2002), and as a consequence, nitrogen could become less limiting in nature and this may counterselect $N_2$-fixing prokaryotes. Will some of them disappear without ever been known? After more than a century of research on $N_2$ fixation, there are still ambitious goals to achieve.

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