11 Dinitrogen-Fixing Prokaryotes

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Abstract
Dinitrogen fixation is a key process in the N cycle and only carried out by few prokaryotes. Research on dinitrogen fixation includes basic and practical applications: from *nif* genes to crops, with molecular, genetic, ecological, taxonomic, and agricultural approaches used. Nitrogen fixing rhizobia, which have been used in agriculture for over a 100 years, are excellent research models still leading the knowledge of eukaryote-bacteria symbioses. Other less known symbioses of dinitrogen fixing bacteria are reviewed as well as free-living diazotrophs.

Introduction

Dinitrogen fixation, the biocatalytic conversion of gaseous nitrogen (N_2) to ammonium, is an exclusive property of prokaryotes, with only few of them having this capacity. Some are symbionts of eukaryotes from plants and animals to protists. The enzymes responsible for nitrogen fixation are nitrogenases (see section ❯ "Nitrogenase Structure"). 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). Biological nitrogen fixation is the main process of incorporation of N_2 into the biosphere, contributing to 65 % of the total input of N in Earth, or 96 % of the N input derived from natural processes, thus being considered as the main process to life after photosynthesis. 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 (*Glycine max*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), clover (*Trifolium* spp.), and bean (*Phaseolus vulgaris*) obtain substantial amounts of their nitrogen from biological N_2 fixation. Worldwide some 44–66 million metric tons of N_2 are fixed by agriculturally important legumes annually, with another 3–5 million metric tons fixed by legumes in natural ecosystems, providing nearly half of all the N used in agriculture (Postgate 1982; Smil 1999; Newton 2000; Graham and Vance 2003). One of the long-term goals of N_2 fixation research is to select or engineer major cereal crops such as rice (*Oryza sativa*), maize (*Zea mays*), and sugarcane (*Saccharum* spp.), 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, *azote* = nitrogen; *trophs* = eaters: dinitrogen fixers) are found to be associated with the roots of plants where they may 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. Symbiotic associations of diazotrophs with eukaryotes as diatoms, corals, and fungi (reviewed in Kneip et al. 2007) cause these organisms to acquire nitrogen-fixing capabilities; the analyses of these symbionts reveal fascinating adaptations and in few cases the evolution of nitrogen-fixing cyanobacteria to become organelles called spheroid bodies in diatoms (Kneip et al. 2008).

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Industrial fixation of atmospheric N_2 tends to exceed 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 N in soil, marine environments such as oligotrophic oceanic waters (where dissolved fixed-nitrogen content is extremely low; 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 papermaking chemicals (Vaisanen et al. 1998). Nevertheless, the presence of a N_2-fixing bacterium is not evidence for the occurrence of N_2 fixation, as in most cases special conditions as low oxygen or a differentiation process are required for nitrogenase to be expressed.

The usefulness of N_2-fixing bacteria in bioremediation is also being recognized (Suominen et al. 2000; Prantera et al. 2002). Transformation of contaminating polychlorinated biphenyls was obtained with alfalfa inoculated with *Ensifer 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).

Dinitrogen fixers are encountered in bacteria and in some groups of archaea. The list of the phyla containing nitrogen-fixing bacteria is probably still far from complete but enlarging. 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 as-yet-uncultured (Rappe 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).

The reaction of nitrogen fixation may be represented by the equation $N_2 + 8H^+ + 6e^- \rightarrow 2NH_3 + H_2$ and is coupled to the hydrolysis of 16 equivalents of ATP. The high cost of energy may thus explain why nitrogen fixation was lost in many bacterial lineages when not needed. The possession of N_2-fixing genes (see section ❯ "Distribution of Dinitrogen-Fixing Ability Among Prokaryotes") does not confer a selective advantage to bacteria in N-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 heavily fertilized sugarcane plants (Fuentes-Ramírez et al. 1993, 1999; 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). Another effect of adding fixed nitrogen (diminished genetic diversity of *Rhizobium* from bean nodules) was observed when the plants were treated with the recommended level of chemical nitrogen (Caballero-Mellado and Martínez-Romero 1999).

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 N-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 N-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. Some *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).

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.

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 (*Triticum aestivum*) 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).

Free-living diazotrophs are capable of fixing N_2 without a host plant, in general utilize the energy available in the environment, and do not excrete or excrete only part of the NH_3 produced. In general, these microorganisms live in the rhizosphere, but others live inside stems, roots, and leaves and therefore are considered as endophytes. In a closer relationship, the symbiotic bacteria use the energy derived from the plant photosynthesis and export the NH_3 synthesized to the host plant. The major contribution to the biological N_2 fixation process occurs by the mutualistic association with plants belonging to the Leguminosae (=Fabaceae) family. This is one of the largest families of plants, with over 18,000 species classified into around 650 genera, representing approximately a twelfth of all known flowering plants and occupying nearly all terrestrial biomes. The family is divided into three subfamilies: *Papilionoideae*, *Mimosoideae*, and *Caesalpinioideae*. Most grain legumes are classified into the subfamily *Papilionoideae*, and 97 % of the species within this family are capable of fixing nitrogen, while in the *Mimosoideae* and *Caesalpinioideae*, the percentages are lower (Allen and Allen 1981; Polhill and Raven 1981; Giller 2001).

Methods for Detecting Nitrogen Fixation

The methods used to quantify plant-associated biological N_2 fixation have been recently reviewed (*Unkovich* et al. 2008) and they include (1) methods related to the analysis of total plant nitrogen, applied to both the nitrogen balance method and the nitrogen difference method; (2) quantification by the ureide-N method in some tropical legumes as soybean and common bean; (3) methods related to ^{15}N isotope, including the ^{15}N-natural abundance, and the ^{15}N isotope dilution; and (4) the acetylene reduction assay. Some of these methods are also used to measure the same process in free-living diazotrophs and bacteria associated with other eucaryotic hosts. N balance method, in which N increases are quantified, is a reliable method if inputs and losses of N are correctly accounted, and the N difference method uses the comparison with a neighbor non-fixing plant species (Unkovich et al. 2008). The ureide method is based on the principle that several legumes (e.g., soybean, bean) transport most of the fixed N_2 as ureides (allantoin and allantoic acid), and evaluation of these compounds in comparison to total N (in the xylem sap or in the petiole) will relate to the quantity of N_2 fixed (Herridge and Peoples 1990; Unkovich et al. 2008). Several experiments have shown positive correlation between the N-ureide and total plant N or grain yield (Neves and Hungria 1987; Hungria et al. 2006a). The ^{15}N-based techniques have also been thoroughly reviewed (Bergersen 1980; Hardarson and Danso 1993), and one of the advantages is the precise quantification with the use of stable isotopes, therefore not requiring special licenses for the laboratory; however, the use of proper controls in the ^{15}N-natural abundance is very important. The acetylene reduction assay (ARA) has been used for over 30 years to measure nitrogenase activity and as an indicator of N_2 fixation (Hardy et al. 1968). The method is based on the principle of alternative substrates of the nitrogenase, one of them acetylene, that is reduced to ethylene that can be easily analyzed by chromatography. Acetylene reduction has been very useful to detect new N_2-fixing microorganisms. However, it has been demonstrated that disturbance of soil, roots, and nodules and of the interface root-soil may drastically affect activity of both nodulated legumes and associative N_2-fixing systems (Minchin et al. 1986; Hunt and Layzell 1993; Unkovich et al. 2008). Alternatives have been proposed, as the evaluation in flow systems and of the H_2 flux, but the assay has not been used for quantification purposes (Unkovich et al. 2008). Novel approaches that use ^{15}N with mass spectrometry (MIMS, multi-isotope imaging mass spectrometry or nano-SIMS) allow the detection of nitrogen fixation in cells in microbial consortia or inside eukaryotic host cells (Lechene et al. 2007; Dekas et al. 2009). These approaches may displace older methods. Using nano-SIMS and fluorescence in situ hybridization nitrogen fixation was found to occur in deep-sea sediments such as in the cold methane seeps that were unknown to be sites for nitrogen fixation (Dekas et al. 2009).

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 anchors to design primers for the synthesis of the nitrogenase genes by means of polymerase chain reaction [PCR] amplification) in the structural *nif* gene sequences (see section "Distribution of Dinitrogen-Fixing Ability Among Prokaryotes"), namely, *nifHDK*, found in many microorganisms (Dean and Jacobson 1992; Ueda et al. 1995). However, finding nitrogenase genes is not evidence that nitrogen-fixation activity occurs. 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. Furthermore, with his approach, the description and natural histories of communities of N_2-fixing microorganisms may be established more accurately than with traditional microbiological techniques. For example, the nitrogenase reductase (*nifH*) genes may be amplified by PCR 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). In other cases, homologous or heterologous probes have been used in

hybridization experiments to detect N_2 fixers. 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). A microarray with a diversity of oligonucleotide corresponding to *nifH* genes has been used to detect diazotrophs (Zhang et al. 2007).

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. Since knowledge of the nitrogenase gene diversity has improved (over 27,600, 950, and 400 *nifH, nifD*, and *nifK* sequences, respectively, were available in the GenBank database 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.

A different method of N_2-fixation detection involves the growth of indicator non-N_2-fixing organisms in a coculture 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 N-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, N_2 fixation could have evolved in methanogenic archaea and subsequently transferred into the bacterial domain (Raymond et al. 2004). Among the domain Archaea, nitrogen fixers (or strains containing nitrogenase genes) are restricted to the phylum Euryarchaeota (Fig. 11.1). Few out of the over 100 currently identifiable major lineages or phyla within the domain Bacteria have nitrogen-fixing members, namely, Proteobacteria, Cyanobacteria, Chlorobi, Spirochaetes, the Gram-positives (Firmicutes and Actinobacteria), Bacteroidetes, Nitrospirae, and Verrucomicrobia, and probably also some strains within Fusobacteria, Deferribacteres, and Fibrobacteres for which only nitrogenase genes have been detected without evidence of nitrogen fixation (Fig. 11.1).

Dinitrogen-fixing organisms have an advantage over non-fixers in N-deficient but not in N-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 (called nodulation genes, including *nod*, *nol*, and *noe* genes) may be easily lost concomitantly with the symbiotic plasmid (Segovia et al. 1991). Similarly, nonsymbiotic *Mesorhizobium* strains, that lack a symbiotic island, are found in nature (Sullivan et al. 1996). On the other hand, acquisition of the whole set of symbiotic genes by nonsymbiotic rhizobia in N-depleted soils has been reported in both *Mesorhizobium* (Sullivan et al. 1995; Sullivan and Ronson 1998) and *Ensifer* (=*Sinorhizobium*) (Barcellos et al. 2007). 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 (*Klebsiella pneumoniae* from clinical specimens) had this capacity (Martínez 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 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

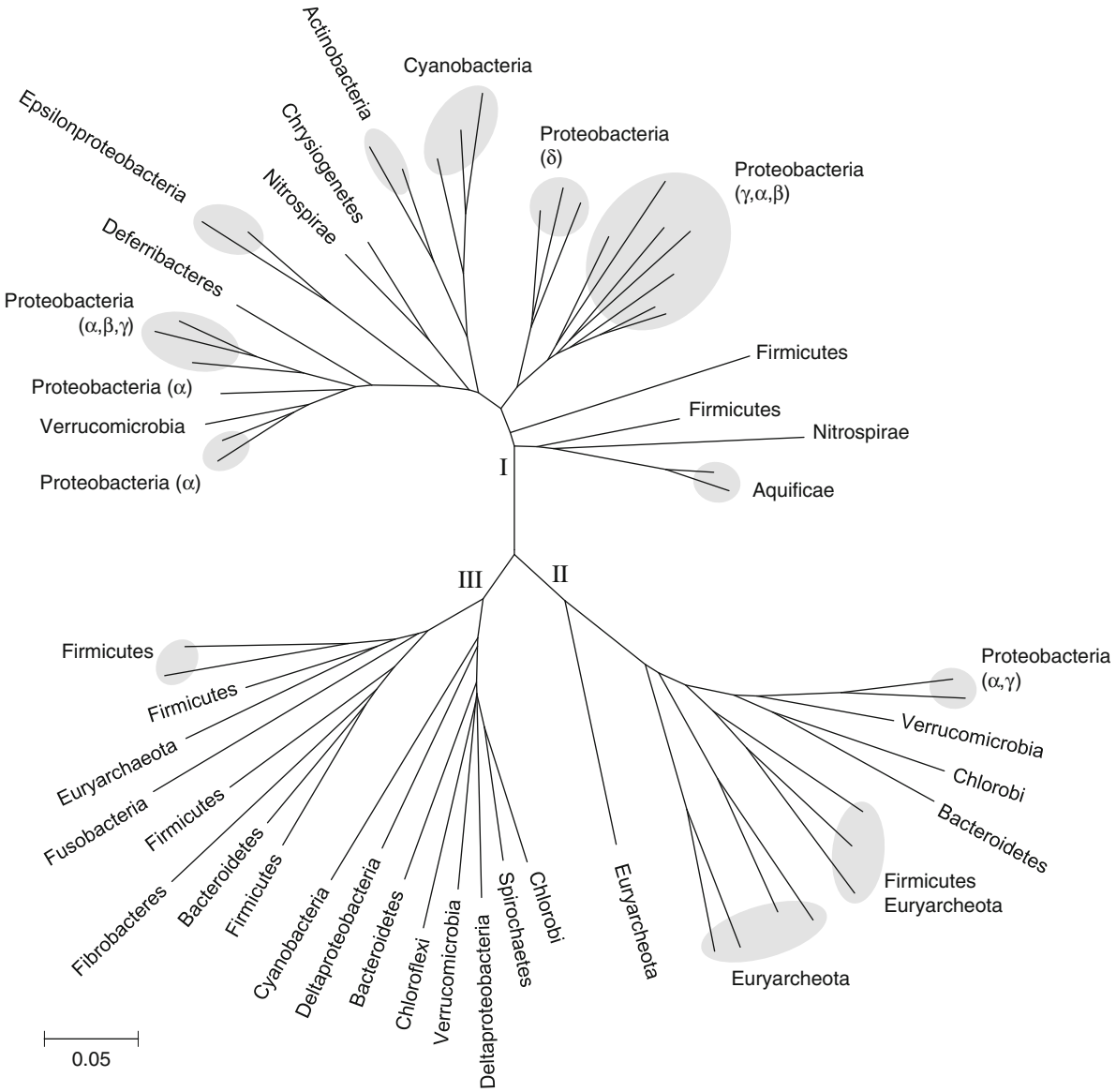

Fig. 11.1
Phylogeny of *nifH* genes encountered in different phyla. *nifH* clades are indicated with Roman numerals

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 *nifH* 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).

The first report on the physiology of N_2 fixation within the phylum Verrucomicrobia of which most members remain

uncultured showed that *Methylacidiphilum fumariolicum*, a metanotroph, fixes nitrogen at low oxygen concentration and the activity was not inhibited by ammonium. Phylogenies of *nifHDK* genes showed that the genes cluster with those from the Proteobacteria (Khadem et al. 2010).

Ecology of Dinitrogen-Fixing Prokaryotes

Free-Living Diazotrophs

The fluctuations of marine diazotroph populations have been analyzed. 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 do not fix nitrogen as efficiently as the nonheterocystous cyanobacteria at the high temperatures of the tropical oceans (Staal et al. 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).

Symbiotic Diazotrophs

The ability to form symbiosis may determine the long-term success of an organism. Nitrogen-fixing symbioses range from rather loose to permanent, characterized by morphological and physiological modifications.

Dinitrogen-fixing cyanobacteria form symbioses with diverse hosts such as fungi, bryophytes, cycads, mosses, ferns, and an angiosperm, *Gunnera* (Bergman et al. 1992). In lichens, sponges, and corals (bipartite or tripartite), cyanobacteria fix nitrogen. Heterocysts, the specialized cyanobacteria cells where nitrogen fixation occurs, are formed by *Nostoc punctiforme* at the hyphal tips of the fungus *Geosiphon pyriformis*, a relative of AM fungi. Epiphytic moss in subtropical moist forest seemingly associated with nitrogen-fixing bacteria showed acetylene reduction activity (Han et al. 2010). The identity of the nitrogen-fixing species is unknown and N fixation should be evaluated with other methods.

Research on the symbiosis of cyanobacterial species and hosts, *Nostoc-Gunnera*, *Richelia-Rhizosolenia*, and *Hemiaulus* showed that some bacterial symbionts may be acquired from the environmnent while others are transmitted from one generation to the next; the luggage endosymbiont hypothesis was reviewed (Wouters et al. 2009). Each host diatom harbors a unique endosymbiotic *Richelia* strain.

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).

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. 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, 1996). 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 *Mesorhizobium loti*, the symbiotic genes are chromosomally located as in most *Mesorhizobium* and *Bradyrhizobium* sp.

The range of nodulating bacteria has enlarged. Nodulating *Methylobacterium* has been reported from *Crotalaria* nodules (Sy et al. 2001). Strains of *Devosia*, *Ochrobactrum*, *Aminobacter*, *Microvirga*, *Shinella*, and *Phyllobacterium* have also been shown to be root-nodule legume symbionts (Rivas et al. 2002; Valverde et al. 2005; Zurdo-Pinero et al. 2007; Estrella et al. 2008; Lin et al. 2008; Ardley et al. 2011). Surprisingly, some Betaproteobacteria in the genera *Burkholderia* (Moulin et al. 2001) and *Cupriavidus* (formerly known as *Ralstonia*) (Chen et al. 2001) are capable of nodulating legumes. Like *Rhizobium* and *Sinorhizobium* spp., some 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).

Nitrogen Fixation in Insects

Estimates are that the contribution of insect-borne nitrogen-fixing bacteria may be up to 30 kg of N/hectare (ha)/year (Nardi et al. 2002).

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 *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).

Primitive termites have protists in their guts that contain bacterial symbionts, among them, one very aggressive wood-eating species *Coptotermes formosus* contains a Bacteroidete that may not be cultured in the lab, designated Candidatus *Azobacteroides pseudotrichonymphae* that has both nitrogen-fixing and cellulolytic capabilities. These bacteria *nifH* genes resemble those from termite gut spirochaetes (Hongoh et al. 2008) and not those from Chlorobi that is a closer relative to Bacteroidetes. Previously no nitrogen-fixing bacteria were recognized among Bacteroidetes, so this is a new phylum in the list of phyla with nitrogen-fixing bacteria.

Enterobacteria mediate nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. Nitrogen-fixing enterobacteria such as *Klebsiella* and *Enterobacter* have been cultured from the fruit fly (Behar et al. 2005).

Nitrogen is a limiting factor not only in human agriculture but also in ant agriculture. Symbiotic nitrogen fixation was detected in the fungus gardens of leaf-cutter ants. Nitrogen-fixing genes detected were found similar to those from *Enterobacter* (Pinto-Tomas et al. 2009) or *Klebsiella variicola* (Rosenblueth et al. 2004).

Other Nitrogen-Fixing Symbioses in Eukaryotes

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). Imaging with mass spectrometry (MIMS) showed that the bacterial symbionts within the wood-eating marine bivalve *Lyrodus pedicellatus* fix nitrogen in host cells. Evidence was also presented that the fixed nitrogen was transferred to the host (Lechene et al. 2007). In sea urchins *Strongylocentrotus droebachiensis*, the nitrogen-fixing activity depended on the diet. Digestive traces exhibited nitrogenase activity (Guerinot et al. 1977).

Symbionts from the pinnate diatom *Rhopalodia gibba* have *nif* genes that resemble both in sequence and organization those genes from free-living cyanobacteria specially *Cyanothece* sp. Remarkably, the cyanobacterial symbiont has gone through a genome reduction process and lost photosynthetic pigments among many other genes. The endosymbiont is no longer photosynthetic, and this function may be supplied by the host. *R. gibba* has not been observed without the bacterial symbiont, and the symbiont does not grow alone in culture media in relation to having a reduced genome (Kneip et al. 2008).

Dinitrogen-Fixing Prokaryotes in the Oceans

Dinitrogen fixation in the world oceans was reviewed by Karl et al. (2002). In a deep-sea hydrothermal vent, a methagenomic archaeal was capable of fixing nitrogen at 92°C (Mehta and Maross 2006). This raises by 28°C the upper limit of temperature for nitrogen fixation. Archaeas in consortia with sulfate reducing bacteria are responsible for nitrogen fixation in the deep ocean sediments (Dekas et al. 2009). These "Fantastic Fixers" (Fulweiler 2009) were functionally detected by nano-SIMS that is useful to detect nitrogen fixation in situ.

A low representation of nitrogen-fixing gene sequences was found in the metagenomic analysis of the ocean (Johnston et al. 2005) that may be explained by the sampling process with elimination of large size cells that would exclude Cyanobacteria as *Trichodesmium* and *Synechococcus*. In coastal sediments, considerable levels of nitrogen fixation have been detected (Fulweiler et al. 2007).

Filamentous cyanobacteria including *Trichodesmium* and *Katagnymene* were the most abundantly detected in an approach independent of culture using real-time quantitative PCR (Langlois et al. 2008). The distribution of *nifH* phylotypes was in relation to water temperature. Unicellular uncultured cyanobacteria were diverse and also abundant. The diatom endosymbiont *Richelia* (Wouters et al. 2009) is considered together with *Trichodesmium* a major nitrogen-fixing bacterium in the ocean (Davis and McGillicuddy 2006). However, there are other marine diazotrophs that have been reported including Proteobacteria (Hewson et al. 2006; Man-Aharonovich et al. 2007) that seem to be widely distributed.

High nitrate concentration does not seem to select against all diazotrophs (Karl et al. 2002; Langlois et al. 2008). In the ocean, iron and phosphate (Sañudo-Wilhelmy et al. 2001) seem to limit

nitrogen fixation (Voss et al. 2004). Available phosphate limits N_2 fixation not only in agricultural fields but also in the ocean (Sañudo-Wilhelmy et al. 2001).

Dinitrogen-Fixing Prokaryotes in Agriculture

Inoculants

History of Inoculants and Inoculation

The use of legumes in agriculture because of their properties of improving soil fertility dates back from a long time, and one example is given by the Romans that developed the idea of crop rotation with legumes and nonlegume plants to improve soil "health" and quality. However, only in 1813, the interest in chemistry of Sir Humphrey Davy led him to report that the legumes "seemed to prepare the ground for wheat" and speculated that the N came from the atmosphere. In 1838, Boussingault, a French agricultural chemist proved that legumes had higher N levels than cereals and concluded that the atmosphere was the source of this N. Finally, in 1886, two German scientists, Hellriegel and Wilfarth, demonstrated that the ability of legumes to convert N_2 from the atmosphere into compounds which could be used by the plant was due to the presence of swellings or nodules on the legume root and to the presence of bacteria within these nodules. The first rhizobia were isolated from nodules soon after, in 1888, by the Dutch microbiologist Martinus Beijerinck, and shown to have the ability to reinfect the legume host and to fix N_2 in symbiosis. Two years later, two German scientists, Nobbe and Hiltner, demonstrated the advantages of adding pure bacteria with the seeds at sowing and soon after submitted the first patent describing the use of artificial inoculation. Finally, in 1898, the first commercial inoculant industry, Nitragin, was established in USA (Voelcker 1896; Fred et al. 1932; Smith 1992; Hungria and Campo 2004; Hungria et al. 2005b).

Determining the Need for Inoculation

Nowadays, the contribution of the biological N_2 fixation process to the N balance in all ecosystems is fully recognized, but it is also known that the inputs are even more critical in the tropics, where the soils have low organic matter and nutrient content and are frequently subject to erosion or inappropriate farm management. In these areas, nutrient depletion may be accentuated by the high cost of fertilizers, especially N sources, the majority of which are imported from developed countries (Hungria and Vargas 2000; Giller 2001; Graham and Vance 2003). For example, Sanchez (2002) estimated an annual average depletion rate of 22 kg of N/ha across 37 countries in Africa. Soil physicochemical exhaustion may lead to depletion of diazotrophic bacteria. Concomitantly, in developing countries, high application of chemical fertilizers also results in constrains of diazotrophic bacteria. Therefore, in both cases, there is need to improve the availability, quality, and delivery of diazotrophic bacteria to the plants, by means of the inoculation process.

Although diazotrophic bacteria are spread in all ecosystems, in several cases, there is need to improve biological N inputs by the addition of inoculants. The practice of inoculation dates from long before the description of the N_2 fixation process, with reports of the importance of transferring soil from a field where legumes have been grown to new areas being planted to the same crop to achieve good establishment. One example was the recommendation in England, in the sixteenth and seventeenth centuries, of transferring soil from established pastures of alfalfa (*Medicago sativa*) to new areas. But it was only after the isolation of the first rhizobia and the establishment of the first inoculant industry that the use of inoculants launched worldwide (Fred et al. 1932; Smith 1992; Hungria and Campo 2004). The main situations where inoculation is needed include the following: (1) when an exotic legume is introduced into a new area where it has not been previously grown and where there is no compatible rhizobia (e.g., soybean in the USA); (2) in soils depleted of diazotrophic bacteria because of different agricultural applications (e.g., high application of chemical N fertilizers); (3) when the soil contains a large population of rhizobia that are compatible, competitive but ineffective with the legume of interest (e.g., several reports with common beans); and (4) when improved strains are needed to sustain highly productive improved cultivars (e.g., modern soybean genotypes). In any of these cases, simple trials can define the need for inoculation and must include a minimum set of three treatments: (1) non-inoculated control without N fertilizer, (2) non-inoculated control with N fertilizer, and (3) inoculated without N fertilizer. The comparison of these three treatments will indicate if the indigenous population is effective in fixing N_2 or if there is need for inoculation, and it will also indicate if the indigenous or inoculant strains are as effective as the N fertilizer or if strain improvement should take place.

Inoculant Production and Utilization

Some aspects related to inoculant production and inoculation will be mentioned in this chapter, and valuable complementary information can be obtained in other reviews (Brockwell and Bottomley 1995; Balatti and Freire 1996; Lupwayi et al. 2000; Stephens and Rask 2000; Catroux et al. 2001; Date 2001; Singleton et al. 2002; Hungria et al. 2005b; Sessitsch et al. 2002).

Inoculants consist of a product carrying the desirable diazotrophic strains in an adequate substrate. In general, desirable properties of a good inoculant carrier can be summarized as follows: (1) readily available, uniform in composition, and cheap in price; (2) nontoxic to the bacteria; (3) with high water retaining capacity; (4) easily sterilized; (5) readily corrected to a final pH of 6.5–7.3 or to a pH adequate to the bacteria; and (6) allowing good initial growth of the target organism and the maintenance of high cell numbers during storage (Hungria et al. 2005b).

Peat has been the most suitable carrier for inoculant production, since it usually meets these requirements, but different

sources of peat vary in their capacity to support rhizobial multiplication and survival (Roughley 1970; Balatti and Freire 1996; Maier and Triplett 1996; Hungria et al. 2005b). Unfortunately, mycobacteria that may represent a risk to human health have been found in peat (De Groote et al. 2006). In the absence of peat, a number of materials fitting the characteristics of a good carrier have been alternatively used with different degrees of success, including vegetable oils, mineral oils, plant materials such as bagasse, silk cocoon waste, sawdust, rice husk, corncob, various clays including vermiculite, perlite mixed with humus, diatomaceous earth, lignite and derivatives, coal, filter mud and charcoal-bentonite, among others (Hungria et al. 2005b). Granular inoculants have also been broadly and successfully used in some countries, as Australia, for soil inoculation, but disadvantages rely on the use of non-sterile substrate and on the lower number of cells per g in comparison to other carriers. For seed inoculation, there are also gels and lyophilized inoculants but representing a low percentage of the market. Worldwide, there is an increase in the number of liquid formulations, due to the facility of sterilization and to the low cost of production (Hungria et al. 2005b). For example, in Brazil, peat inoculants represented 98 % of a market of 12 million doses in 1999, shifting to a market of 60 % of liquid inoculants of a total of 18.7 million doses in 2009. Liquid inoculants must carry cellular protectors, usually proprietary substances to protect the rhizobia. Alginate, xanthans, Fe-EDTA, trehalose, carboxymethyl cellulose (CMC), polyvinylpyrrolidone (PVP), and a variety of other polymers have been listed among the bacteria protectors. Simple procedures of manufacturing liquid inoculants have been developed and may be applied for small-scale procedures. An interesting example was achieved in a project coordinated by Niftal (Singleton et al. 2002), and other procedures are listed by Balatti and Freire (1996).

Sterilization is an expensive but key step, because a sterile carrier allows high concentration of cells; furthermore, it reduces the frequency and level of contamination and thus the risk of introducing and disseminating plant, animal, and human pathogens (Lupwayi et al. 2000; Catroux et al. 2001; Hungria et al. 2005b). In countries with high-quality products, sterile products are the general rule or are mandated by legislation, and concentration of cells in inoculation is established in at least 10^8 cells/g or mL of the product, but with the technology available today, there are several products with concentrations as high as 10^{10} cells/g or mL. Nevertheless, despite the biotechnological advances achieved in the past decades, unfortunately, a considerable percentage of the inoculants produced in the world are still of relatively poor quality (Brockwell and Bottomley 1995; Lupwayi et al. 2000; Stephens and Rask 2000; Hungria et al. 2005b).

Nitrogen Fixation with Legumes

In natural ecosystems, the rates of N_2 fixation are sufficient to attend the needs of nodulated plants, but commercial crops require far greater amounts of N. Consequently, to achieve high yields, inoculation with selected strains is often mandatory. Quantification of N_2 fixation is a difficult task (Unkovich et al. 2008), but in the studies performed so far with several legumes, the estimates of the contribution of the biological process to the plant N nutrition may be very impressive (*Table 11.1*).

Exotic grain legumes broadly used as commercial crops need inoculation with compatible rhizobia. One relevant example is that of the soybean, a crop with top priority in South America (Hungria et al. 2005a), USA (Paau 1989), and in many other countries where inoculation is considered a realistic alternative to the increasing use of fertilizers. When soybean was introduced in South America, inoculation was needed to guarantee nodulation and N_2 fixation, thus in the 1960s and 1970s, strains were brought from foreigner countries to guarantee grain production in the absence of chemical fertilizers. As the soils were void of natural compatible rhizobia, a new approach was considered in the strain selection program and consisted of isolating and testing hundreds of strains after a period of establishment on the soils. The selection process has shown that it was possible to identify natural variants adapted to the tropical conditions and more efficient and competitive than the parental strains. Concomitantly, the plant breeding program has always considered the symbiotic performance with selected strains used in commercial inoculants. This approach has been very successful, such that high soybean grain yields are obtained in Brazil based exclusively on the N_2-fixation process (Hungria and Vargas 2000; Hungria et al. 2005a, 2006a, b). Noteworthy is the economy related to the N_2 fixation with the soybean crop in Brazil, estimated at US $6.6 billion/year.

The specific requirement of inoculants of most commercial soybean varieties can be attributed to the breeding for grain yield conducted largely in North America in the 1960s and 1970s and then spread to other countries. However, in areas where inoculants are not readily available, soybean growth was limited; promiscuous varieties capable of nodulating with indigenous rhizobia were then developed in a breeding program performed by IITA in Africa (Pulver et al. 1985) and were also identified from early introductions of soybean originated from China (Mpepereki et al. 2000). Nowadays, there are reports that some of the promiscuous genotypes can also respond to inoculation, and efforts have been applied to improve yields of these genotypes. In parallel, more productive non-promiscuous genotypes have been introduced in Africa and will need inoculation.

Soybean can also associate with fast-growing strains that were first isolated from soybean nodules and soil from the People's Republic of China, within the center of origin and diversity of this legume (Keyser et al. 1982); the majority of these strains are classified as *Sinorhizobium* (=*Ensifer*) *fredii*. Some of the fast-growers can be very effective in fixing N_2 with the soybean, with successful reports of responses to inoculation in Spain, but the main limitation relies in that *S. fredii* can only outcompete *Bradyrhizobium* at high pH, of 7 or 8 (Buendía-Clavería et al. 1994; Hungria et al. 2001).

Reinoculation is an important topic to be considered both in the case of exotic plants growing in soils that have been previously inoculated and show an established or naturalized

Table 11.1
Estimates of N_2 fixation rates with some legumes. In most cases, the values represent the compilation of several studies

Species	Common name	N_2 fixation rates (kg N/ha)	Reference
Acacia spp.	Acacia	5–50	Sprent and Parsons (2000)
Arachis hypogaea	Groundnut	32–206	Unkovich and Pate (2000)
Cajanus cajan	Pigeon pea	68–88	Giller and Wilson (1991)
Calopogonium muconoides	Calopogonium	64–182	Giller and Wilson (1991)
Centrosema spp.	Centrosema	41–280	Giller and Wilson (1991)
Cicer arietinum	Chickpea	0–141	Unkovich and Pate (2000)
Desmodium spp.	Desmodium	25–380	Giller and Wilson (1991)
Gliricida sepium	Gliricida	26–75	Giller and Wilson (1991)
Glycine max	Soybean	0–450	Unkovich and Pate (2000); Hungria et al. (2005a)
Lathyrus sativus	Lathyrus	172–227	Unkovich and Pate (2000)
Lens culinaris	Lentil	5–191	Unkovich and Pate (2000)
Leucaena leucocephala	Leucaena	98–274	Giller and Wilson (1991)
Lupinus albus	Sweet lupin	40–160	Unkovich and Pate (2000)
Lupinus angustifolius	Lupin	19–327	Unkovich and Pate (2000)
Lupinus mutabilis	Bitter lupin	95–527	Unkovich and Pate (2000)
Macroptilium atropurpureum	Siratro	46–167	Giller and Wilson (1991)
Medicago sativa	Alfalfa	45–470	Unkovich and Pate (2000); Russelle and Birr (2004)
Melilotus officinalis	Yellow sweet clover	84	Unkovich and Pate (2000)
Neonotonia wightii	Perennial soybean	126	Giller and Wilson (1991)
Phaseolus vulgaris	Common bean	0–165	Giller and Wilson (1991); Unkovich and Pate (2000)
Pisum sativum	Field pea	4–244	Unkovich and Pate (2000)
Pueraria phaseoloides	Tropical kudzu	115	Giller and Wilson (1991)
Sesbania spp.	Sesbania	7–109	Giller and Wilson (1991)
Stylosanthes spp.	Stylosanthes	4–263	Giller and Wilson (1991)
Trifolium spp.	Clover	67–260	Unkovich and Pate (2000)
Vicia benghalensis	Vetch	125–147	Unkovich and Pate (2000)
Vicia faba	Faba bean	12–330	Unkovich and Pate (2000)
Vigna mango	Black gram	119–140	Giller and Wilson (1991)
Vigna radiata	Green gram	58–107	Giller and Wilson (1991)
Vigna unguiculata	Cowpea	9–201	Giller and Wilson (1991)
Zornia glabra	Zornia	61	Giller and Wilson (1991)

population or in the case of legumes compatible with indigenous strains. Inconsistent responses to inoculant application are frequently attributed to these naturalized/indigenous rhizobia, to the enrichment of populations with the cropping of legumes, or to a combination of both factors (e.g., Sadowsky and Graham 1998; Thies et al. 1991; Thies et al. 1995). There are reports of declines in the response of soybean to inoculation when the numbers of rhizobial cells are as low as 10–20 cells/g of soil (Weaver and Frederick 1974; Singleton and Tavares 1986; Thies et al. 1991). However, with the use of selected strains and high-quality inoculants, responses to soybean reinoculation have been reported in soils with 10^3 cells/g of soil or higher, of up to 10^6 cells/g of soil. In 74 field trials performed in soils with high population of soybean bradyrhizobia in Argentina, yield was enhanced by a mean of 14 % in comparison to the non-inoculated treatment, while in 29 field experiments performed in Brazil, reinoculation increased yield by 8 % (Hungria et al. 2006b). Most important, in all of these experiments, there was no response to the application of N fertilizers in any stage of plant growth (Hungria et al. 2006a). Several mutants of soybean cultivars, known as supernodulators and tolerant to nitrate have been obtained (Carroll et al. 1985; Gremaud and Harper 1989; Akao and Kouchi 1992), but they also require inoculation with *Bradyrhizobium* strains.

For indigenous legumes, or legumes that have been grown for a long time in the area, e.g., common bean in Africa and Brazil, responses to inoculation can be erratic. Taken as example common bean, poor nodulation and lack of responses to

inoculation in field experiments have been frequently reported worldwide (Graham 1981; Hardarson 1993; Vlassak and Vanderleyden 1997). Explanation for the failure in those trials would rely mainly on a high population of competitive indigenous rhizobia but with low efficiency of nitrogen fixation (Graham 1981; Thies et al. 1991). In addition, common bean-rhizobia symbiosis is quite sensitive to environmental stresses, such as high temperatures and soil dryness, leading to low N_2-fixation efficiency (Graham 1981; Hungria and Vargas 2000). However, it has been shown that the search—within the natural diversity of indigenous soil population—for effective and competitive strains can be successful. In Brazil, the use of selected strains of *Rhizobium tropici* has resulted in increases in grain yield of up to 900 kg/ha. Noteworthy is that the responses of common bean to inoculation with elite strains in Brazil were observed in soils with at least 10^3 cells/g of compatible rhizobia (Hungria et al. 2000b, 2003).

A variety of other economically important legumes has important contributions to the N inputs. One important example of legume forage is alfalfa (*Table 11.1*), estimated to be one of the five most valuable crops in the world, with a worth value estimated in US 7 billion/year (Howieson et al. 2008). 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 rate of fixation of the tree *Acacia dealbata* is considered sufficient to replace the estimated loss due to timber harvesting (May and Attiwill 2003), and despite the low number of studies performed so far, some long-term trials had proved that inoculation can improve biomass production of trees (Lal and Khana 1996). The application of green manure can also contribute with high inputs of N. 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 *Rhizobium huautlense* is also a flood-tolerant symbiosis (Wang and Martínez-Romero 2000). Despite not representing a legume-rhizobia symbiosis, the so-called actinorhizal plants that associate with *Frankia* should also be mentioned, as they are of great value for reforestation; actinorhizal plants belong to eight families (Baker and Mullin 1992; Berry 1994).

From the values of N_2 fixation reported so far, one may conclude that the contribution of N_2 fixation with legumes is fundamental to the global N cycling and to recover and maintain soil fertility. This contribution can be substantially increased by the inoculation with selected strains delivered in high-quality inoculants. Unfortunately, the adoption of legumes in agricultural systems is still low, even in countries that need higher contribution, e.g., estimates in African farming systems are that less than 5 % of area planted to legumes (Giller et al. 2006). However, the prospects for the use of legumes in a variety of new applications are outstanding (Howieson et al. 2008). Concerns should be raised about a higher use of N fertilizers, decreasing the capacity of N_2 fixation (van Kessel and Hartley 2000). On the other hand, there are research and extension areas in which in-depth efforts were needed to maximize the contribution of N_2 fixation to agriculture: (1) plant improvement (breeding); (2) to alleviate and avoid constrains related to acid soils, soil acidification, soil degradation, desertification, and salinization; (3) to search for new legume genotypes; (4) to allow adequate phosphorus supply and utilization; (5) to stimulate crop rotation; and (6) to improve strains and inoculants (Graham and Vance 2000; Howieson et al. 2008). Unfortunately, despite the great advances achieved in the last decade in the knowledge of diazotrophic bacteria, with an emphasis on the "omics" studies, the field constrains to the biological process have increased, and the efforts toward plant and strain breeding for improved N_2 fixation are scarce. 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.

Nitrogen Fixation with Nonlegumes

A high impact goal of nitrogen-fixation research has been to extend the process to nonlegumes, and this has promoted the search for diazotrophic bacteria that are associated with agriculturally valuable crops. From a basic research perspective, this has increased our knowledge of their diversity. A critical review of the actual contributions of bacterial N_2 fixation to the amount of 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 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 400 kg N/ha (*Table 11.1*), while conservative values for bacterial fixation in nonlegumes are 20–30 kg N/ha per year, but higher, substantial values have been also estimated.

N_2 fixation with nonlegumes has started with research with *Azospirillum*, dating back to the pioneering work of Dr. Johanna Döbereiner (Döbereiner and Day 1976; Döbereiner et al. 1976). After that, many reports have shown that *Azospirillum* may promote N_2 fixation, growth, and yield of numerous plant species, many of which are of agronomic or ecological importance (e.g., Okon and Labandera-Gonzalez 1994; Bashan and Holguin 1997; Hungria et al. 2010). Inoculants containing *Azospirillum* have been tested under field conditions with important crops in developing and developed countries, with various degrees of responses. In a survey of 20 years of experiments, Okon and Labandera-Gonzalez (1994) reported that 60–70 % of the experiments showed yield increases due to inoculation with

Azospirillum, with statistically significant increases in yield from 5 % to 30 %. In Argentina, in a survey of 273 cases of inoculation of wheat with *Azospirillum brasilense*, 76 % resulted in a mean yield increase of 256 kg/ha; with maize, 85 % of the cases were successful, resulting in a mean yield increase of 472 kg/ha (Díaz-Zorita and Fernandez Canigia 2008). In addition, the colonization and contribution of several other rhizospheric and endophytic N_2-fixing bacteria have been broadly reported.

Sugarcane (*Saccharum* spp.), rice (*Oriza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*) are the Gramineae most extensively studied with regard to N_2 fixation, but other crops are being studied as well. Sugarcane has been grown for more than 100 years in some areas of Brazil without N fertilization or with very low N inputs, and removal of the total harvest has not led to decline in yield and soil N levels. This observation suggested that N_2 fixation may have been the source for a substantial part of the N used by this crop (Döbereiner 1961). From 25 % to 55 % (Urquiaga et al. 1989; Yoneyama et al. 1997) or perhaps as much as 60–80 % (Boddey et al. 1991) of the sugarcane, N could be derived from associative N_2 fixation, but skepticism about the occurrence of high levels of N_2 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 N uptake by different sugarcane varieties (Urquiaga et al. 1989), declining ^{15}N enrichment of soil mineral N, carryovers of N from one harvest to the next, and differential effects on control plants during the studies (Urquiaga et al. 1992). The mean estimates of fixed N_2 for two sugarcane hybrids grown in concrete tanks ranged from 170 to 210 kg N_2 fixed/ha (Urquiaga et al. 1992), and evidences of large differences in N_2 fixation among different sugarcane cultivars are compelling. Correction for micronutrient soil deficiencies and high soil moisture seems to be key conditions that promote N_2 fixation in sugarcane plants (Urquiaga et al. 1992).

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 Döbereiner 1988), *Herbaspirillum* (Baldani et al. 1986), and *Burkholderia* (Oliveira et al. 2009). Probably, N_2 fixation in sugarcane is performed by a bacterial consortium (Oliveira et al. 2009). *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 wild-type 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 have also 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). *G. diazotrophicus* (James and Olivares 1997), *Herbaspirillum seropedicae*, and *Herbaspirillum rubrisubalbicans* (Olivares et al. 1996) have been clearly shown to colonize sugarcane plants internally. Colonization by *G. diazotrophicus* was inhibited by N fertilization (Fuentes-Ramírez et al. 1999).

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), among other countries. These studies report a positive balance with estimates of around 16–60 kg of N/ha/crop (App et al. 1986; Ladha et al. 1993). In a N 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 (Boddey et al. 1995; Watanabe et al. 1987).

Many different N_2-fixing bacteria have been isolated from rice roots. These include *Azotobacter*, *Beijerinckia* (Döbereiner 1961), *Azospirillum* (Baldani and Döbereiner 1980; Ladha et al. 1982), *Pseudomonas* (Qui et al. 1981; Barraquio et al. 1982, 1983; 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 been long used to fertilize agricultural land throughout the world, most notably rice paddies in Asia. Increases in rice plant growth and increases in N 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 (Van Trân et al. 1996). *B. 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

(Van Trân 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).

N_2-fixing bacteria associated to maize include *Azospirillum*, *Herbaspirillum*, *Klebsiella* (Chelius and Triplett 2001), *B. vietnamiensis* (Van Trân et al. 1996), *Rhizobium etli* (Gutierrez-Zamora and Martinez-Romero 2001), *Paenibacillus brasilensis* (von der Weid et al. 2002), and *Klebsiella variicola* (Rosenblueth et al. 2004). *K. 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 da Silva et al. 2003).

In a survey performed by Sumner (1990), thirty-two experiments with cereals were considered to respond positively to inoculation, but there were some negative responses, mostly in wheat. However, positive responses of wheat to inoculation with *Azospirillum* have been reported, resulting in increase in grain yield of up to 18 % in Brazil (Hungria et al. 2010) and up to 63 % in Mexico (Caballero-Mellado et al. 1992). However, in both studies, the main benefits were attributed to plant-growth promotion properties, and not to N_2 fixation.

There are an increasing number of reports on the isolation of diazotrophic rhizospheric and endophytic bacteria from several plant species. Sweet potato (*Ipomoea batatas*) may grow in soils poor on N, and associated N_2 fixation has been considered to contribute N to these plants. By a cultivation-independent approach, bacteria similar to *Klebsiella*, *Rhizobium*, and *Ensifer* were inferred to be present as sweet potato endophytes (Reiter et al. 2003). Several diazotrophic genera have also been isolated from important crops as cassava (*Manihot esculenta* Crantz) (Balota et al. 1997), coffee (*Coffea arabica* L.) (Jimenez-Salgado et al. 1997), flowers, and fruits, among others. An elucidation of the mechanisms related to the quantitative and qualitative differences of diazotrophic bacteria associated with plants is still missing, and probably, several factors may be related, among them the carbon sources released by the plants (e.g., Christiansen-Weniger et al. 1992).

Interface Rhizobia-Associative/Endophytic Bacteria

In nature, several plant species compose the different ecosystems, and in traditional agriculture, plants are grown in crop rotation, succession, or intercropped. It is thus expected that several diazotrophic bacteria may be hosted by different plant species. For over seven centuries, rice rotation with clover (*Trifolium* spp.) 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 were also encountered inside the rice plant with around 10^4–10^6 rhizobia/g (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.

In nonlegumes (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, 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 N content as compared to non-inoculated 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 too low to maintain bacterial growth and N_2 fixation (Welbaum et al. 1992; Fuentes-Ramírez et al. 1999). In acreage cultivated using *S. rostrata*-rice rotation, *A. caulinodans* survives in the soils and rhizosphere of wetland rice (Ladha et al. 1992). *A. caulinodans* can colonize the rice rhizosphere (specifically around the site of lateral root emergence), can penetrate the root at the site of emergence of lateral roots, and can colonize subepidermally intercellular spaces and dead host cells of the outer rice root cortex (Reddy et al. 1997).

Growth stimulation of crops such as wheat and maize inoculated with a *R. leguminosarum* bv. *trifolii* strain may not be related to N_2 fixation (Hölflich et al. 1995). In Mexico, *R. etli* was found to colonize maize genotypes (Gutierrez-Zamora and Martinez-Romero 2001). On the other hand, a compilation of studies of co-inoculation of rhizobium with azospirillum has shown benefits in parameters as expression of nodulation genes, nodulation, nitrogenase activity, plant biomass and root growth in chickpea (*Cicer arietinum*), field pea (*Pisum sativum*), soybean, common bean, common vetch (*Vicia sativa*), faba bean (*Vicia faba*), black medick (*Medicago polymorpha*), alfalfa (*Medicago sativa*), winged bean (*Psophocarpus tetragonolobus)*, and white clover (*Trifolium repens*) (Dardanelli et al. 2008). A better understanding of the complex interactions of associative, endophytic, and symbiotic diazotrophic bacteria with plants should be gained in the next years through approaches as metagenomics.

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 a 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 (nitrogenase 2), and the iron-only nitrogenase (nitrogenase 3). 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, N-free media. The V-containing nitrogenase produces around three times more hydrogen than the Mo nitrogenase (Eady 1996).

As a result of the reduction of N_2 by the nitrogenase, H_2 is obligatorily produced, resulting in a lost of energy and electrons of at least 25 %. However, some bacteria possess a second enzyme, hydrogenase, capable of oxidizing the H_2, producing ATP, and recovering part of the energy lost in the process (Evans et al. 1985, 1987). 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* 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, 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). Some endophytes have been found to release (excrete) riboflavin during N_2 fixation (Phillips et al. 1999). 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 (Hardy and Havelka 1973; Zanetti et al. 1996).

Nitrogenase Structure

The classical nitrogenase is a complex, two-component metalloprotein composed of an iron (Fe) protein and a molybdenum-iron (MoFe) protein. 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, and it is the active site of substrate reduction (Hoover et al. 1989). All substrate reduction reactions catalyzed by nitrogenase require the sequential association and dissociation of the two nitrogenase components.

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 *A. 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

Nitrogenase genes seem to have gone through a long period of divergence and genes from the most conserved of the nitrogenase genes, *nifH* fall into different families (http://www.es.ucsc.edu/~wwwzehr/research/database/). 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. 11.2*). 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 formation 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),

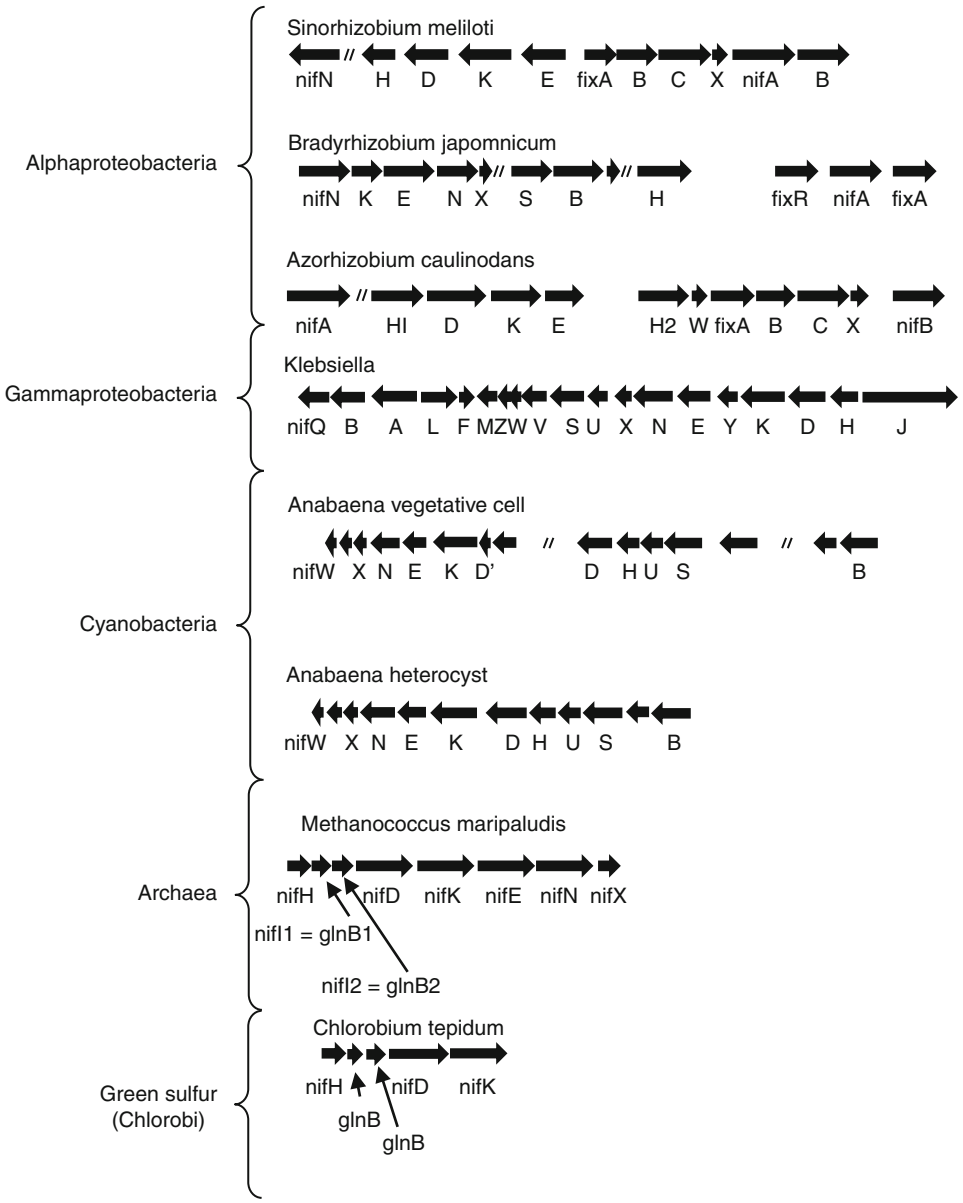

Fig. 11.2

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

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. 11.2*).

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 low substrate specificity might have occurred before the last common ancestor of all living organisms emerged (Fani et al. 1999).

The repeated sequences clustered around the *nif* region of the *B. 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).

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).

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. Cyanobacteria and Archaea have 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).

Different regulatory elements 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 very complicated regulatory cascades (Dixon 1998; Nordlund 2000; Forchhammer 2003; Zhang et al. 2003). Very diverse modes of regulation of *nif* genes have been described that vary between species (D'hooghe et al. 1995; Girard et al. 2000). Detailed studies have been carried out in *K. pneumoniae*, *A. vinelandii*, *A. brasilense*, *R. capsulatus*, *Rhodospirillum rubrum*, *Sinorhizobium meliloti*, or *B. japonicum*. 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). 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), *B. 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).

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 σ^N (σ^{54}) and the transcriptional activator NifA (Kustu et al. 1989). Regulators of NifA vary among different diazotrophs. 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).

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 (*Ensifer*, *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*), differences in the fine mechanisms regulating N_2 fixation exist and have been reviewed (Fischer 1994; Kaminski et al. 1998).

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 downregulated by ammonium or nitrate (Thiel et al. 1995; Muro-Pastor et al. 1999).

Lessons from Genomics

Genome size and organization among nitrogen-fixers can vary widely. The genome of the cyanobacteria *Nostoc* (which is a symbiont of cycads, *Gunnera* and others) is among the largest from prokaryotes, with nearly 10 Mb (Meeks et al. 2001). In contrast the genome of the *Azolla* cyanobacterium symbiont has suffered a large reduction (Ran et al. 2010), the true *Azolla* symbionts are non-culturable. The sequences of the genomes of the legume-nodulating bacteria belonging to the genera of *Mesorhizobium*, *Ensifer* and *Bradyrhizobium* revealed contrasting chromosome sizes and highly diverging genomes. Large genomic differences have been found as well among *Frankia* genomes from different plant hosts (Normand et al. 2007).

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 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 2000) but has been detected in other arqueae.

The genome sequence of the Bacteroidete Candidatus *Azobacteroides pseudotrichonymphae* from the guts of old termites revealed nitrogen-fixing genes (Hongoh et al. 2008). Genes for nitrogen fixation were discovered in the *Fusobacterium nucleatum* genome, no members of the Fusobacteria phylum were previously known to fix nitrogen. Similarly the genomes of Candidatus *Nitrospira defluvii*, of *Fibrobacter succinogenes* and *Deferribacter desulfuricans* revealed nitrogen-fixation pathways, for these to our knowledge, there was no evidence yet of nitrogen-fixation activity. These bacteria are the first representatives of their own phylum to be described as nitrogen-fixing species.

The genome analysis of the chlorinated-ethene-respiring *Dehalococcoides ethenogenes* strain 195 showed the presence of a complete nitrogenase operon and subsequently the nitrogen fixation was demonstrated by incorporation of ^{15}N and transcription of *nifD* gene (Lee et al. 2009). Interestingly this is a strain that belongs to the phylum Chloroflexi previously known to fix N_2. Out of 4 sequenced strains only *Dehalococcoides ethenogenes* contained *nif* genes and these resembled *Clostridium* and archaea *nif* genes, all anaerobic.

Hydrogenobacter thermophilus strain TK-6 belongs to the family Aquificaceae (Yoshino et al. 2001), in a the phylum Aquificae (one of the earliest branching in Bacteria) and has the *nifH* gene but *nifDK* are pseudogenes.

Non-cultured insect endosymbionts can be studied by genomic aproaches; the reduced genome from the spheroid body from *Rhopalodia gibba* has revealed its close relatedness to free-living nitrogen-fixing cyanobacterium (Kneip et al. 2008).

Conclusions and 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. 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 an ambitious goal is feasible is difficult to predict. The modification of cereals such as rice to render them capable of forming nodules is being explored based on the large knowledge of symbiotic genes in model legume plants (Rolfe et al. 1998).

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 from associated bacteria is being considered as a suitable mode to exploit N_2 fixation in nonlegumes.

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 and proteomics 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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