CHAPTER 15

Biological Dinitrogen Fixation: Introduction and Nonsymbiotic

David A. Zuberer

> *In nature there is an enormous reserve of organic matter poor in nitrogen. One should ask how all this organic carbon could be circulated in nature without the existence of organisms capable of fixing free nitrogen. These organisms . . . could be nothing else but microbes.*
>
> —Sergei Winogradsky, 1895

After photosynthesis, biological dinitrogen fixation—the reduction of atmospheric dinitrogen (N_2) to two molecules of ammonia—is the second most important biological process on earth. In the absence of modern fertilizers or animal wastes, natural ecosystems rely on the biological conversion of atmospheric dinitrogen to forms available for plant and microbial growth by a variety of prokaryotic microbes. Dinitrogen fixation is mediated exclusively by prokaryotes, including many genera of bacteria (Chapter 5), cyanobacteria (Chapter 7), and the actinomycete *Frankia* (Chapter 16). The N_2-fixing microbes can exist as independent, free-living organisms or in associations of differing degrees of complexity with other microbes, plants, and animals. These range from loose associations, such as **associative symbioses**, to complex **symbiotic** associations (Fig. 15–1) in which the bacterium and host plant communicate on an exquisite molecular level and share physiological functions (Chapter 16 details information on symbiotic N_2-fixing plant/microbe associations). Central to all of these systems is an N_2-fixing prokaryote containing the enzyme complex **nitrogenase**, which is responsible for the conversion of dinitrogen to ammonia. Only certain prokaryotes fix dinitrogen. Thus, when one sees statements about "N_2-fixing" plants, keep in mind that they do so by virtue of their prokaryotic partner, not on their own. Organisms that can use atmospheric dinitrogen as their sole source of nitrogen for growth are called **diazotrophs** ("diazo" for dinitrogen).

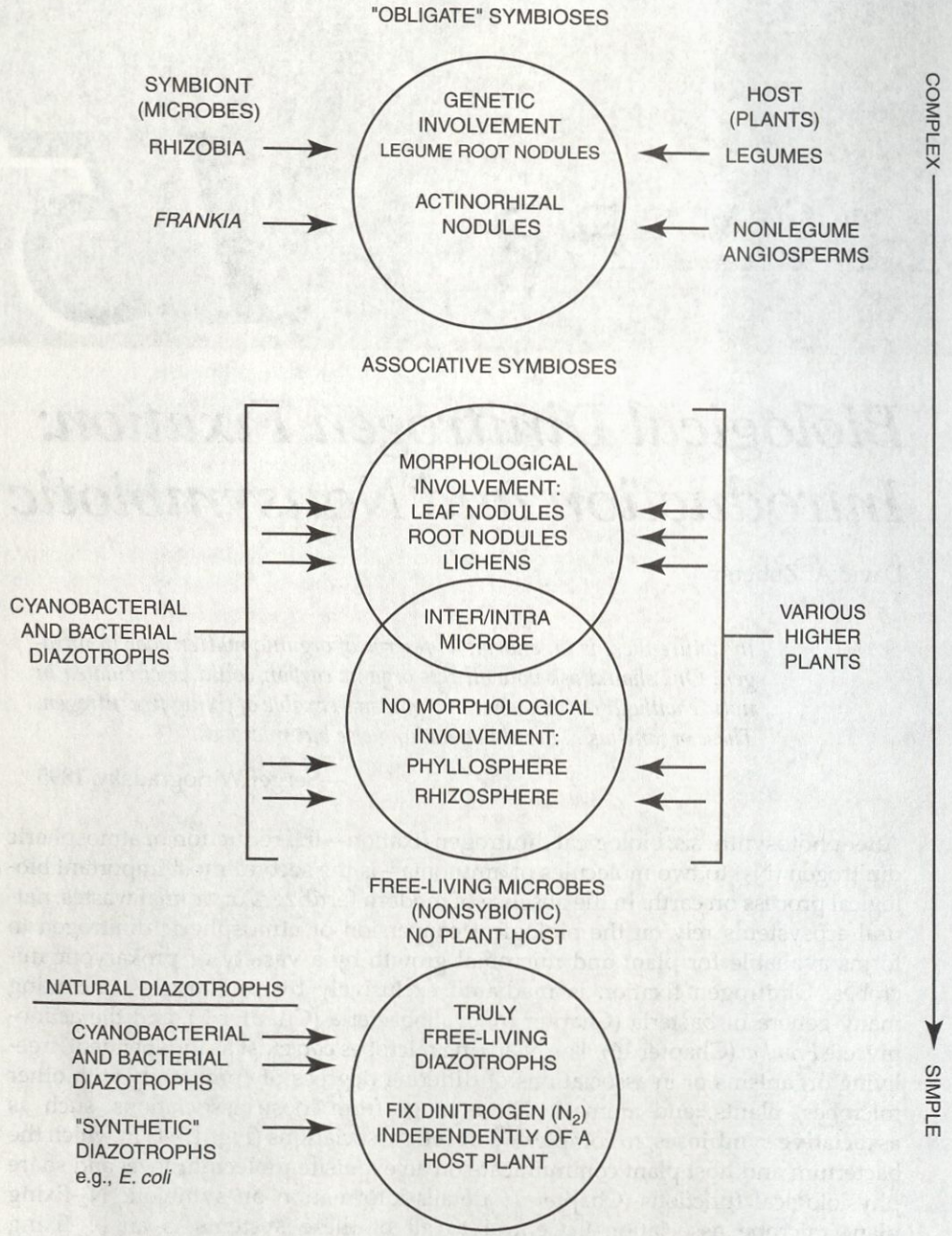


FIGURE 15–1
Biological N_2 fixation is mediated exclusively by free-living prokaryotic diazotrophs and a wide variety of plant-microbe associations of varying complexities. The most elaborate of these are the root-nodule symbioses between rhizobia and legumes and between the actinomycete *Frankia* and a variety of nonleguminous trees and shrubs. Between the free-living diazotrophs and the root-nodule symbioses lie a broad array of associations between plants and diazotrophs. These are the so-called associative symbioses characterized by a loose association between the plant and diazotroph. *Modified from Burns and Hardy (1975). Used with permission.*

HISTORICAL BACKGROUND

The agricultural importance of legumes was recognized very early in the history of humans, reaching a milestone in 1888 with the isolation and description of the first organism, the root-nodule bacterium *Rhizobium*, determined (though much later) to have the ability to fix dinitrogen. *Rhizobium* and its relatives fix only small amounts of dinitrogen in pure culture, and significant rates of N_2 fixation only occur when the bacteria are in root nodules (Chapter 16). In 1893, Winogradsky isolated the first free-living bacterium, an anaerobe, capable of significant N_2 fixation. He named this organism *Clostridium pasteurianum* in recognition of Louis Pasteur; Winogradsky, though a Russian, did much of his later scientific work at the Pasteur Institute in Paris. A few years later, in 1901, the Dutch microbiologist Martinus Beijerinck isolated an aerobic free-living, N_2-fixing bacterium, which he named *Azotobacter chroococcum* (derived from the French word "azote," meaning "lifeless" in recognition of the inertness of dinitrogen).

Thus by the late nineteenth century, the foundation was laid for the extensive studies of N_2 fixation that would follow. These early discoveries began what has become more than 100 years of research on the fascinating and important subject of biological N_2 fixation.

THE SIGNIFICANCE OF BIOLOGICAL DINITROGEN FIXATION

A fundamental principle of agriculture and plant physiology is that plants require relatively high levels of nitrogen to produce abundant biomass, or yield. All forms of life require nitrogen to synthesize proteins and other important biochemicals, and nitrogen is often the limiting nutrient for plant and microbial growth in soils. In natural systems, nitrogen for plant growth comes from the soil, from rainfall or other atmospheric deposition, or through biological N_2 fixation.

Industrial N_2 fixation amounts to about 85 million metric tons per year (Hauck, 1985; Waggoner, 1994) and requires substantial inputs of energy, usually in the form of natural gas (Box 15–1). However, this output is less than the contribution of fixed nitrogen acquired through biological N_2 fixation. Accurate estimates of the magnitude of biological N_2 fixation are hard to derive, but reported values range from 100 to 180 million metric tons per year. Biological processes contribute 65% of the nitrogen used in agriculture (Burris and Roberts, 1993). Though much of this is through symbiotic N_2 fixation (Chapter 16), nonsymbiotic and associative N_2 fixation are of some significance in crops, such as sugarcane and sorghum, which have a C_4 photosynthetic pathway, and in specific ecosystems where nitrogen for plant growth is a limiting factor. Because of its culture in flooded soils, rice derives significant benefit from the activities of free-living diazotrophic bacteria and cyanobacteria.

Biological N_2 fixation offers an alternative to the use of expensive ammonium-based fertilizer nitrogen. However, the high-yielding agricultural systems of the United States and elsewhere are difficult to sustain solely on biological N_2 fixation. Keeping up with an expanding population will probably always require the judicious

BOX 15–1

The Energy Costs of N_2 Fixation

Dinitrogen is described as the most stable diatomic molecule known. The two atoms of nitrogen in the diatomic molecule are joined by a very stable triple bond. This triple bond requires a lot of energy (945 kJ, or 226 kcal, per mole) to break, and therein lies one of the major challenges of fixing dinitrogen chemically or biologically. Dinitrogen fixation is "energy expensive" because it requires much energy to break the triple bond of dinitrogen and also to provide the hydrogen necessary to reduce dinitrogen to two ammonia molecules. The chemical fixation of dinitrogen is accomplished most widely through the high-pressure catalytic method called the Haber-Bosch process, named after the German scientists who developed the process in 1914. The process served as a source of ammonium and nitrates for manufacture of explosives during World War I. The reaction for chemical fixation of dinitrogen can be written as follows:

$$N_2 + 3H_2 \xrightarrow[K_2O,\ Al_2O_3]{} 2\,NH_3\,(aq.)\ \Delta G = -53kJ\ (12.7kcal)$$

To obtain high yields from this reaction in reasonable time, high pressures (approximately 200 atm. or 20 MPa) are applied to the reaction vessel and the temperature is raised to 400–500°C. Natural gas (methane) is usually employed as the feedstock for the hydrogen needed to reduce the dinitrogen and is also required to heat the reaction vessel. To put these energy requirements in terms more easily understood, it takes about 875 cubic meters (31,000 cubic feet) of natural gas, 5.5 barrels of oil, or 2 metric tons of coal to fix 1 metric ton (2,200 lb) of ammonia (Dixon and Wheeler, 1986). About 40% of this fuel is used to provide the necessary heat, and the remainder provides the hydrogen needed for the reaction. Because we rely on fossil fuel (e.g., natural gas) in the production of ammonium-based fertilizers, costs are high; at present, those costs are somewhat inextricably linked to fossil fuel prices and the whims of that market. For example, an oil embargo against the United States in the early 1970s brought about long lines at gas pumps and quadrupled fertilizer prices. The high fertilizer prices also were due, in part, to short supplies at a time of peak demand.

Just as the chemical fixation of dinitrogen is energy intensive, so too is its fixation in biological systems. The principal differences lie in the sources of reductant and energy, and the fact that biological N_2 fixation takes place at ambient pressures and temperatures. That is quite a feat when we consider the rigors of the industrial process. Energy for biological N_2 fixation comes from the oxidation of organic carbon sources, such as glucose, or from light in the case of photosynthetic diazotrophs.

use of fertilizers. Yet, legumes and perhaps other N_2-fixing systems (e.g., the *Azolla-Anabaena* symbiosis) have an important place in sustainable agricultural production. Dinitrogen-fixing plants are also of high value in restoring disturbed or impoverished soils. They serve as excellent cover crops, green manures, and forage crops for livestock production. Use of N_2-fixing crops also has the potential to reduce the contamination of groundwater with nitrate. Further aspects of the utility of symbiotic N_2 fixation in agriculture and other ecosystems are discussed in Chapter 16.

E NITROGENASE ENZYME COMPLEX

The process of biological N_2 fixation is restricted, so far as known, to prokaryotic microbes including many genera of soil bacteria, cyanobacteria (formerly the "blue-green algae"), and a few actinomycetes (most notably *Frankia*). These microbes can fix dinitrogen as **free-living** forms (i.e., not plant associated), in loose associations with higher plants called **associative symbioses** (e.g., many grass-bacteria associations) (Fig.15–1), and in truly symbiotic partnerships with higher plants (the **root-nodule symbioses** involving rhizobia and *Frankia*).

A trait shared by all these microbes is that they produce the enzyme complex called **nitrogenase** which mediates biological N_2 fixation. It is most appropriately called the "nitrogenase complex" because it is actually composed of two protein components each composed of multiple subunits. The nomenclature of the nitrogenase complex is as follows (Evans and Burris, 1992):

- The overall complex is known as **nitrogenase**.
- The molybdenum-iron (MoFe) protein is **dinitrogenase** (the "type species" substrate is dinitrogen; enzymes conventionally are named relative to their substrate).
- The iron (Fe) protein is designated **dinitrogenase reductase**; the general consensus is that its function is the reduction of dinitrogenase.

Characteristics of the two proteins are summarized in Table 15–1. A diagrammatic representation of the nitrogenase complex is shown in Figure 15–2. What sets the nitrogenase complex apart is that it:

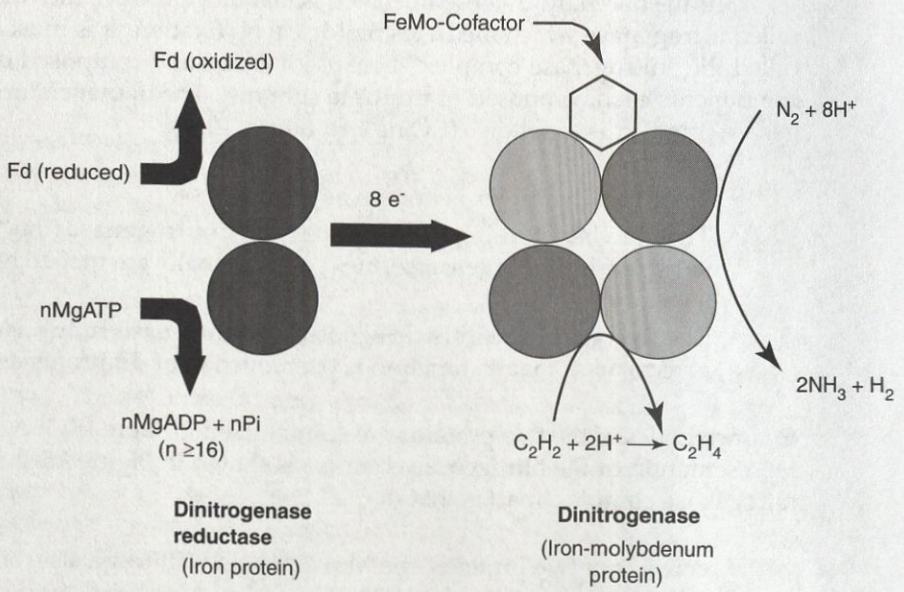
- consists of two proteins, the MoFe protein (dinitrogenase) and the Fe protein (dinitrogenase reductase),
- is destroyed by oxygen,
- contains iron and molybdenum or vanadium,
- needs Mg^{2+} ions to be active,
- converts ATP to ADP when functioning,
- is inhibited by ADP,
- reduces dinitrogen and several other small triply bonded molecules, and
- reduces H^+ to H_2 even when dinitrogen is present.

TABLE 15–1 Characteristics of Dinitrogenase (the MoFe Protein) and Dinitrogenase Reductase (the Fe Protein)

Dinitrogenase
- Mol. wt.: 220,000–270,000
- Has 4 subunits: 2 approximately 50,000 mol. wt.
  2 approximately 59,000 mol. wt.
- 2 molybdenum atoms per molecule
- 21–35 iron atoms per molecule (depending on species)
- 18–24 (labile sulfur) atoms per molecule (depending on species)
- Half-life in air: up to 10 min.

Dinitrogenase reductase
- Mol. wt.: 55,000–66,000
- Has 2 subunits: 27,500–34,000
- No molybdenum
- 4 iron atoms per molecule
- 4 labile sulfur atoms per molecule
- Half-life in air: 0.5–0.75 sec.

Adapted from Postgate (1998). Used with permission.



Overall reaction:

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

FIGURE 15–2
The nitrogenase enzyme complex consists of two protein components (each with multiple subunits): dinitrogenase reductase (the Fe protein) and dinitrogenase (the MoFe protein). The dinitrogenase reductase gathers electrons from low-redox carriers such as ferredoxin and flavodoxin and passes them on to the dinitrogenase protein. Nitrogen is believed to be bound to the dinitrogenase protein where it is reduced.

The overall reaction for biological N_2 fixation using nitrogenase is shown in Figure 15–2. Two MgATP are required for each electron transferred from dinitrogenase reductase to dinitrogenase; thus the reaction, as written, shows a requirement of 16 molecules of ATP (112 kcal). Under natural conditions, however, probably 20–30 MgATP are needed as the process is less efficient than when observed under optimum laboratory conditions (Burris and Roberts, 1993). A consensus on the general model for the mechanism of nitrogenase has evolved over quite a few years. The mechanism can be summarized as follows:

- Dinitrogenase reductase (the Fe protein) accepts electrons from a low-redox donor such as reduced ferredoxin (Fd_{red}) or flavodoxin and it binds two MgATP (Fig. 15–2).
- It transfers electrons, one at a time, to dinitrogenase (the MoFe protein).
- Dinitrogenase reductase and dinitrogenase form a complex, the electron is transferred, and two MgATP are hydrolyzed to two MgADP + Pi (phosphate).
- Dinitrogenase reductase and dinitrogenase dissociate and the process is then repeated.
- When dinitrogenase has collected enough electrons, it binds a molecule of dinitrogen, reduces it, and releases NH_4^+.
- The MoFe protein (dinitrogenase) then accepts additional electrons from dinitrogenase reductase (the Fe protein) to repeat the cycle.

In each cycle of N_2 fixation, dinitrogenase and dinitrogenase reductase bind together, MgATP is hydrolyzed, and an electron is transferred. The dissociation of the MoFe protein–Fe protein complex is the rate-limiting step of the process. In fact, the nitrogenase complex is "remarkably slow—it takes 1.25 sec for a molecule of enzyme to form two of NH_3. The two proteins have to come together and separate 8 times to reduce one dinitrogen molecule" (Postgate, 1998, p. 20). A consequence of the slowness of nitrogenase is that N_2-fixing bacteria must synthesize a lot of the protein. Nitrogenase can commonly account for 10% of the cell's proteins and levels up to 40% have been recorded (Postgate, 1994).

Returning to the reaction in Figure 15–2 for biological N_2 fixation, we can observe that for every $8e^-$ transferred via the nitrogenase complex, $2e^-$ are consumed in the formation of H_2. The production of H_2 that accompanies the fixation of N_2 is obligatory. One H_2 (requiring 4 MgATP) is released for each N_2 reduced to $2NH_3$ (requiring 12 MgATP). Thus, 25% of the energy from MgATP is "lost" in the production of H_2. Interestingly, some diazotrophs contain an uptake hydrogenase that allows them to oxidize some of the H_2 and to generate a reduced electron carrier or MgATP. This can then be used in the N_2 fixation reaction thereby recapturing some of the energy lost.

The physiological requirements for a free-living diazotroph to fix N_2 are summarized in Table 15–2. These requirements stem in large part from the unique properties and requirements of the nitrogenase complex including its exceptional sensitivity to molecular oxygen, the metal content of the complex (iron and

TABLE 15–2 Physiological Requirements for Dinitrogen Fixation by Free-Living Diazotrophic Microbes

Trace elements
Molybdenum or vanadium, iron — for nitrogenase
Magnesium — for production of MgATP

ATP—a minimum of 16 ATP per N_2 fixed. Probably 20–30 under natural conditions. However, *Azotobacter* may use as little as 6 ATP per N_2 fixed.
Needed for nitrogenase activity and for nitrogenase synthesis.
The high ATP requirement means an abundant supply of energy-yielding substrates must be readily available for vigorous N_2 fixation.

Minimize H_2 evolution—up to 35% of the ATP diverted to nitrogenase may be consumed in H_2 evolution.

Acceptable temperature
Most diazotrophs are mesophiles.
Many will not grow on media at 37°C.
Nitrogenase activity falls off rapidly at about 40°C.

Oxygen excluded from the enzyme complex
Nitrogenase is destroyed by O_2

Source of low-redox reductant
Restricted to the naturally occurring ferredoxins and flavodoxins.

Adapted from Postgate (1998). Used with permission.

TABLE 15–3 Substrates for Nitrogenase: The Triple Bond Connection

Name	Formula	Major Products
Proton	H^+	H_2
Dinitrogen	$N{\equiv}N$	$NH_3 + H_2$
Nitrous oxide	$N{\equiv}N^+{-}O^-$	$N_2 + H_2O$
Azide	$[N{\equiv}N^+{-}N]^-$	$N_2 + NH_3 + N_2H_4$
Acetylene	$HC{\equiv}CH$	$H_2C = CH_2$
Cyanide	$[C{\equiv}N]^-$	$CH_4 + NH_3$
Carbon monoxide	$C{\equiv}O$	$C{\equiv}O$ apparently binds to the N-binding site but is not reduced. It blocks reaction with other substrates.

molybdenum or vanadium), and the need for adequate supplies of reducing power and MgATP.

Substrates for Nitrogenase

The principal substrate for nitrogenase is dinitrogen ($N{\equiv}N$). Note that the two atoms of nitrogen are joined by a triple bond. In addition to reducing protons to H_2, nitrogenase also reduces several other small, triply bonded molecules (Table 15–3).

Of particular interest on this list of substrates is the gas acetylene. In the late 1960s researchers discovered that nitrogenase can reduce acetylene ($HC{\equiv}CH$) to ethylene ($H_2C{=}CH_2$) (Burris, 1974). This finding is significant because acetylene and ethylene can be very easily measured using a gas chromatograph to separate the two gases. Thus was born the simple, sensitive, and rapid **acetylene reduction assay** for nitrogenase activity. The development of this assay led to an explosion in research on N_2 fixation because it removed the need to have an expensive mass spectrometer or to deal with the tedious and insensitive Kjeldahl analyses to measure nitrogen in soil or biological materials. However, the technique is not without limitations, which will be discussed later in the chapter.

The Alternative Nitrogenases

Prior to the early 1980s, researchers thought that only one type of nitrogenase existed and that molybdenum was essential for N_2 fixation. However, in the early 1980s, Bishop and colleagues isolated a second nitrogenase (nitrogenase 2) from *Azotobacter chroococcum*, which was produced only under conditions of molybdenum starvation (Bishop and Premakumar, 1992). With this discovery, it became clear that molybdenum was not required for N_2 fixation in all bacteria and could be supplanted with vanadium. Nitrogenase 2 is similar to nitrogenase 1 because it consists of two proteins (a VFe protein and an Fe protein), produces hydrogen, and is sensitive to oxygen. Nitrogen binds to the VFe protein, suggesting that metals other than molybdenum can participate in binding the nitrogen to the protein for reduction. Subsequently, a third nitrogenase, nitrogenase 3, has been described. This complex does not appear to contain either molybdenum or vanadium and is synthesized under starvation for both of the metals.

All the nitrogenases are complexes of a dinitrogenase reductase and a dinitrogenase component. Nitrogenases 2 and 3 appear to evolve more hydrogen than nitrogenase 1, and nitrogenases 2 and 3 reduce acetylene to ethane (C_2H_6) rather than ethylene. Quite recently, yet another nitrogenase has been described (Ribbe, Gadkari, and Meyer, 1997). It occurs in *Streptomyces thermoautotrophicus*, a unique, thermophilic (it can grow at 65°C), chemoautotrophic actinomycete that can use carbon monoxide (CO) as an energy source, oxidizing it to carbon dioxide, or it can also use hydrogen. It fixes dinitrogen and is unique in that its nitrogenase is not inhibited by carbon monoxide, as are the others, it does *not* reduce acetylene, and it is *not* poisoned by oxygen. Currently, it is believed that the manganese-containing enzyme is in fact a superoxide dismutase that supplies electrons to the molybdoprotein that is the "dinitrogenase." It is astonishing that not only is this nitrogenase not poisoned by oxygen, but, quite strikingly, oxygen is *required* in the reaction mechanism of the enzyme complex.

The finding that dinitrogen could be fixed in the absence of molybdenum is interesting because prior to the discovery of the alternative nitrogenases, microbiologists and biochemists believed that the element was an obligate requirement for N_2 fixation. It now seems that the predominance of nitrogenase 1 (the molybdenum-containing enzyme) may simply be because researchers have routinely used

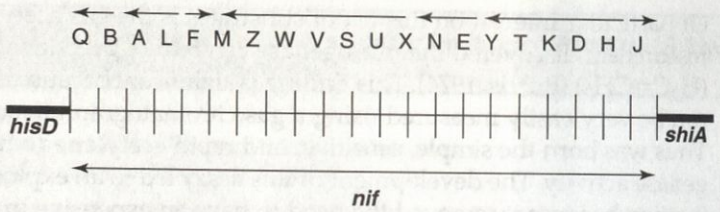


FIGURE 15–3
Map of the N_2 fixation genes of *Klebsiella pneumoniae*. The capital letters denote the separate genes that make up the *nif* cluster. The upper arrows indicate subclusters (operons) in which genes are sequentially transcribed as a group and the directions in which they are read. Brief descriptions of the function(s) of each gene are given in Table 15–4. *Redrawn from Postgate (1998). Used with permission.*

molybdenum-containing media to isolate N_2-fixing bacteria. The other nitrogenases may be quite common in nature, though this possibility has not yet been fully explored. The study of alternative nitrogenase systems is quite recent, and many fundamental questions remain unanswered.

The Genetics of Nitrogenase

As one might surmise from the complexities of the system just described, the genetics of the process are equally complex. Much of our knowledge of the genetics of the nitrogenase system has come from the intensive study of the bacterium *Klebsiella pneumoniae*, a member of the family Enterobacteriaceae and a close relative of *Escherichia coli*. Thirty years of research on this bacterium and, more recently, other diazotrophic bacteria have shown that the nitrogenase complex and supporting systems are under the control of 20 or more genes (Fig. 15–3; Table 15–4). The functions of the N_2 fixation genes are summarized in Table 15–4, starting with the gene on the right-hand side of Figure 15–3. Clearly, the complexity of this system does not make it an easy target for genetic manipulation and transfer to higher organisms. Still, some hold out the prospect that we may someday see genetically engineered crops like corn, wheat, and other cereals that fix their own dinitrogen. The finding of the oxygen-requiring nitrogenase system of *Streptomyces thermoautotrophicus* suggests that the oxygen inhibition of N_2 fixation may not be insurmountable. However, this is one of the more elusive goals facing N_2-fixation researchers.

THE FREE-LIVING DINITROGEN-FIXING BACTERIA

Biological N_2 fixation is restricted to prokaryotes This includes typical bacteria and specialized bacteria like cyanobacteria and the actinomycetes. Of the 10,000 or so bacterial genera named, only about 100 contain bona fide diazotrophic species (Table 15–5). Although this may appear to be a somewhat limited representation, species representing all sorts of physiological types and occupying all sorts of ecological niches have been described. Some of the genera of free-living

TABLE 15–4 The Functions of the Dinitrogen Fixation Genes Are Summarized in Order, Starting with the Gene on the Right Hand Side of Fig. 15–3

nif J codes for an enzyme (pyruvate oxido-reductase) which generates from pyruvate the electrons used for the reduction of dinitrogen.
nif H codes for a peptide molecule, two of which combine to form dinitrogenase reductase, the smaller of the proteins constituting nitrogenase.
nif D codes for a peptide molecule (called the α-subunit), two of which combine to form part of dinitrogenase, the molybdoprotein of nitrogenase.
nif K codes for a somewhat different peptide molecule (called the β subunit). Two of these combine to form the other part of the dinitrogenase protein, which is thus an $\alpha_2\beta_2$ tetramer composed of two pairs of similar peptides.
nif T codes for a very small protein molecule of unknown function.
Nif Y codes for a protein of uncertain function.
nif E codes for a peptide molecule, two of which combine to form the NifE product.
nif N codes for a peptide molecule, two for which combine to form the NifN product. The NifE and NifN proteins combine together to form a protein somewhat resembling dinitrogenase, which appears to act as a template for synthesizing FeMoco.
nif X codes for a protein which may have a regulatory function.
nif U codes for a protein possibly concerned with the iron-sulfur center of dinitrogenase reductase.
nif S codes for a protein which may also be concerned with the Fe-S center of dinitrogenase reductase.
nif V codes for a peptide, two molecules of which combine to form an enzyme which makes homocitrate, a part of FeMoco, from 2-oxoglutarate and acetyl-coenzyme A.
nif W codes for a protein necessary for full activity of dinitrogenase.
nif Z codes for a protein which seems to be involved in the insertion of FeMoco into dinitrogenase.
nif M codes for a protein concerned with rendering dinitrogenase reductase active.
nif F codes for the flavodoxin which accepts electrons from pyruvate, via the *nif J* product, and passes them to dinitrogenase reductase.
nif L codes for a regulator protein which switches off the whole *nif* cluster.
nif A codes for a regulator protein which switches on the whole *nif* cluster.
nif B codes for a protein involved in the synthesis of FeMoco.
nif Q codes for a protein involved in the uptake of molybdenum for FeMoco synthesis.

Adapted from Postgate (1998). Used with permission.

diazotrophs are listed in Table 15–5. These organisms encompass such groups as heterotrophic and chemoautotrophic bacteria, photoautotrophs (bacteria and cyanobacteria), and photoorganotrophs with respect to carbon metabolism (Chapter 3). Note also that they are well represented by aerobes, **microaerophiles** that grow best at low oxygen tension, facultative anaerobes, and obligate anaerobes. Such metabolic diversity enables some type of diazotroph to colonize almost any imaginable sort of habitat. Indeed, diazotrophs are widespread in nature as free-living microbes and in a large number of associations with plants and animals. This great metabolic diversity means that in all sorts of environments, diazotrophs can make contributions to the supply of fixed nitrogen for growth of nonfixing microbes and higher forms of life. For example, diazotrophs colonize the roots and rhizospheres (Chapters 2 and 11) of many plant species and make small quantities of fixed nitrogen available to the plants.

TABLE 15–5 Genera of Microbes That Include Dinitrogen-Fixing Species or Strains. This List Is Not Intended to Be All-Inclusive

	Genus or Type	Species (Examples Only)
AEROBES	*Azotobacter*	*A. chroococcum,* A. vinelandii**
	Azotococcus	*A. agilis**
	Azomonas	*A. macrocytogenes**
	Beijerinckia	*B. indica,* B. fluminis**
	Derxia	*D. gummosa**
	Pseudomonas	*P. stutzeri, P. saccharophila*
	Azoarcus	*A. communis, A. indigens*
	Gluconacetobacter	*G. diazotrophicus, G. johannae*
FACULTATIVE (aerobic when not fixing nitrogen)	*Klebsiella*	*K. pneumoniae, K. oxytoca*
	Bacillus	*B. polymyxa, B. macerans*
	Enterobacter	*E. agglomerans (Erwinia herbicola)*
	Citrobacter	*C. freundii*
	Escherichia	*E. intermedia*
	Propionibacterium	*P. shermanii, P. petersonii*
	Paenibacillus	*P. polymyxa, P. macerans, P. azotofixans.*
MICROAEROPHILES (normal aerobes when not fixing dinitrogen)	*Xanthobacter*	*X. flavus,* X. autotrophicus*
	Acidothiobacillus	*A. ferrooxidans*
	Azospirillum	*A. lipoferum,* A. braziliensis**
	Aquaspirillum	*A. perigrinum,* A. fascicilus**
	Methylosinus	*M. trichosporum*
	Herbaspirillum	*H. seropedicae*
	Burkholderia	*B. brasilense*
STRICT ANAEROBES	*Clostridium*	*C. pasteurianum,* C. butyricum*
	Desulfovibrio	*D. vulgaris, D. desulficuricans*
	Methanosarcina	*M. barkeri*
	Methanococcus	*M. thermolithotrophicus*
PHOTOTROPHS (aerobic) cyanobacteria	*Anabaena*	*A. cylindrica, A. inaequalis*
	Nostoc	*N. muscorum*
	Calothrix	
	(7 other genera of heterocystous cyanobacteria)	
	Gloeothece	*G. alpicola*
PHOTOTROPHS (microaerophiles) cyanobacteria	*Plectonema*	*P. boryanum*
	Lyngbya	*L. aestuarii*
	Oscillatoria	
	Spirulina	
PHOTOTROPHS (facultative) bacteria	*Rhodospirillum*	*R. rubrum*
	Rhodopseudomonas	*R. palustris*
PHOTOTROPHS (anaerobes) bacteria	*Chromatium*	*C. vinosum*
	Chlorobium	*C. limicola*
	Thiopedia	
	Ectothiospira	*E. shapovnikovii*

*Signifies that all reported strains of the species fix dinitrogen.
Adapted from Postgate (1998). Used with permission. See also Young (1992).

FACTORS AFFECTING DINITROGEN FIXATION BY FREE-LIVING DIAZOTROPHS

Thus far, we have discussed the complexities and uniqueness of the nitrogenase complex. In this section the factors that must be successfully integrated for a nonsymbiotic diazotroph to fix dinitrogen will be discussed, followed by a description of the integration of these factors with respect to the functioning of nonsymbiotic diazotrophs in association with higher plants, the so-called **associative symbioses.**

Sources of Energy for Diazotrophs

With the exception of the phototrophic bacteria and cyanobacteria, all diazotrophs require an organic or inorganic, in the case of chemoautotrophs, energy source. A requirement for abundant energy sources is dictated by the high energy demands of nitrogenase. Remember, a minimum of 16 ATPs are required to make two molecules of ammonia from dinitrogen. A wide variety of carbon sources, ranging from methane (CH_4) to complex carbohydrates, can be used by one diazotroph or another. It is not usually the type of carbon source that limits dinitrogen fixation, but the lack of an ample supply in many habitats that limits fixation by the nonsymbiotic bacteria. The soil is not an "organic soup" abundantly supplied with readily available carbon sources, and diazotrophs must compete with all the other soil microbes for the same carbon.

In terms of carbon sources for energy, it is interesting to consider the efficiency of N_2 fixation according to the amount of carbon consumed per nitrogen fixed (Table 15–6). Note that the efficiencies of N_2 fixation in terms of mg of N fixed g^{-1} of carbon source are low, averaging about 15 mg N g^{-1} of carbon source. Also, the assimilation of ammonium is about twice as efficient as N_2 fixation, and that fixation by anaerobes is generally much less efficient than by aerobes. An exception to this generalization is the reduced efficiency of fixation by *Azotobacter* at higher levels of oxygen. An efficiency of 15 mg g^{-1} of carbon source is equivalent to about 13 kg (30 lb) of nitrogen fixed per ton of carbon source. The need for large amounts of available energy sources is obvious.

In nature, the carbon sources for N_2 fixation by heterotrophic bacteria normally come from the remains of crop residues and roots decomposing in the soil and carbon released from active roots. Addition of readily available carbon sources to soil generally stimulates N_2 fixation for short periods of time.

Effects of Combined Nitrogen on Dinitrogen Fixation

Since N_2 fixation is so costly to a cell, it is not surprising that the nitrogenase complex is under strong regulation by the level of combined nitrogen (i.e., ammonium, nitrate, and organic nitrogen) in the medium or the environment. The formation of

TABLE 15–6 Carbon and Energy Source Requirements for Heterotrophs Grown in Chemostats Limited by the Carbon and Energy Source

Organism	Carbon and Energy Source	Efficiency of Nitrogen Incorporation During Growth (mg N g^{-1} C and Energy Source Used)		Carbon and Energy Expenditure for N_2 Fixation	
		N_2	NH_4^+	(g C g^{-1} N_2)	lb C Used Per 100 lb of N_2 Fixed
Anaerobic growth by fermentation					
Clostridium pasteurianum	Sucrose	11	22	46	4,600 (2,054)*
Klebsiella pneumoniae	Glucose	8	19	72	7,200 (3,214)
Aerobic growth					
Klebsiella pneumoniae (O_2-limited)	Glucose	15	35	38	3,800 (1,696)
Azospirillum brasilense (9 μ*M* O_2)	Malate	26	48	19	1,900 (848)
Azotobacter vinelandii (2–10 μ*M* O_2)	Sucrose	16	63	47	4,700 (2,098)
Azotobacter vinelandii (180 μ*M* O_2)	Sucrose	7	38	117	11,700 (5,223)

*The 100-lb figure is chosen as representative of a typical amount of fertilizer nitrogen that might be applied to a crop. Numbers in ( ) are values in kg.
Adapted from Hill (1992). Used with permission. See also Giller and Day (1985).

the nitrogenase complex is repressed by ammonium at fairly low levels. For example, Alexander and Zuberer (1989a) showed that as little as 4.2 μg ml^{-1} of ammonium nitrogen in nutrient solution eliminated acetylene reduction associated with corn roots at an oxygen concentration of 2% (0.02 atm; 2 kPa) around the roots. Nitrate and organic nitrogen also prevent the synthesis of nitrogenase. By repressing the formation of nitrogenase at very low levels of combined nitrogen the organisms avoid the high expense of synthesizing and operating an enzyme system that is not needed under conditions of nitrogen sufficiency. Additionally, there is a "switch off" mechanism through which ammonia shuts down nitrogenase activity.

Effects of Oxygen on Nitrogenase Activity

An interesting characteristic of nitrogenase, and one that poses considerable problems for most free-living diazotrophs, is its extreme sensitivity to molecular oxygen. In most bacteria the enzyme complex is irreversibly "poisoned" by exposure

to O_2. This oxygen sensitivity has led to the development of some unique strategies among the diazotrophs to protect the enzyme system. Most unusual among these is the ability of some *Azotobacter* species to protect their enzymes through respiratory or conformational protection. Box 15–2 illustrates the many adaptations that allow N_2 fixation to occur in environments with widely divergent aeration conditions. It is apparent from the previous discussion that oxygen plays a significant role in governing the occurrence of N_2 fixation in various environments. It is not surprising that the higher rates of N_2 fixation reported in the literature are generally associated with seasonally or chronically wet soils because here the oxygen levels tend to be lower due to saturation. It is for these reasons that paddied rice is one of the few agricultural crops that derive significant benefit from the activities of nonsymbiotic diazotrophs and N_2-fixing cyanobacteria.

BOX 15–2

How Microbes Solve the Oxygen Problems for Nitrogenase

- *Avoidance:* Anaerobes and facultative anaerobes fix dinitrogen only in the absence of oxygen with the exception of *Klebsiella pneumoniae*, which can tolerate very low levels of oxygen. In fact, oxygen is one of the factors that regulates the synthesis of nitrogenase in this bacterium.
- *Microaerophily:* Most aerobic diazotrophic bacteria fix dinitrogen maximally at low partial pressures of oxygen, thereby lessening the exposure of nitrogenase to oxygen. For example, Okon, et al. (1977) report that *Azospirillum* fixes dinitrogen most rapidly at 0.7 kPa (0.007 atm) oxygen.
- *Respiratory protection:* Respiration functions in all aerobes to divert oxygen away from nitrogenase to some extent. In certain *Azotobacter* species, an exaggerated form of respiratory protection is observed. In fact, this bacterium exhibits one of the highest respiration rates of all life forms. The high respiration rate serves to scavenge oxygen and to keep it away from nitrogenase. As a consequence, the bacterium must consume large amounts of substrate to scavenge oxygen and growth is very inefficient in terms of carbon consumed under these conditions.
- *Production of specialized cells:* Many diazotrophic cyanobacteria produce specialized, thick-walled cells, called **heterocysts,** in which the nitrogenase is compartmentalized. These cells do not evolve oxygen in photosynthesis, and the thick wall excludes external oxygen. The diazotrophic actinomycete, *Frankia*, produces vesicles to protect nitrogenase from oxygen. The vesicle wall becomes thicker as the oxygen concentration increases in the medium.
- *Slime:* Aerobic diazotrophs grown on nitrogen-free agar media frequently produce large, gummy colonies caused by the production of extracellular

BOX 15–2 *(continued)*

polysaccharides. The gum serves as a diffusion barrier to the free flow of oxygen into the colony so that cells in the interior of the colony are not as exposed to oxygen. The efficacy of this mechanism is questionable.

- *Conformational protection:* Some *Azotobacter* species produce a protein that binds to the nitrogenase and protects it from damage by oxygen. In the presence of oxygen and when respiration cannot keep up with the incoming oxygen supply, the protein binds to the nitrogenase complex and alters its conformation (shape) to protect it from oxygen. When exposed to oxygen, the organism stops N_2 fixation abruptly, but when conditions of oxygenation return to a more favorable state, the organism resumes N_2 fixation. The enzyme is not destroyed.
- *Temporal or spatial separation of N_2 fixation and oxygen-evolving processes:* Nonheterocystous cyanobacteria solve the oxygen problem by fixing dinitrogen primarily during the dark phase of growth (i.e., temporal separation) when oxygen evolution is not occurring, and respiration also serves to scavenge oxygen away from nitrogenase. Others form aggregates of cells, and some of these cells then function in a microaerophilic environment with spatial separation.

After Postgate (1998). Used with permission.

Other Environmental Factors

The supply of available carbon, presence or absence of sufficient combined nitrogen, and the abundance of molecular oxygen exert primary control over the synthesis and level of nitrogenase activity expressed by bacteria in a given environment. Other environmental factors are of lesser significance but cannot be ignored. Nitrogenase is active over a fairly narrow temperature range. At the lower limits of 5 to 10°C, nitrogenase activity is low, whereas at the upper limits, 37 to 40°C, nitrogenase activity falls off rapidly because of the sensitivity of the enzyme to heat. Most diazotrophs are mesophiles, but there are a few exceptions. For example, some thermophilic cyanobacteria grow in hot springs. *Methanococcus thermolithotrophicus* (a methanogen) fixes N_2 at 64°C, and the recently discovered *Streptomyces thermoautotrophicus* fixes N_2 at 65°C.

Numerous other factors can affect the growth and survival of diazotrophs and thus directly or indirectly influence N_2 fixation. Among these are adequate supplies of phosphorus (N_2 fixation requires high levels of phosphorus), other inorganic nutrients, especially trace metals, and the acidity or alkalinity of the environment. Biotic factors are also involved (Table 15–7).

TABLE 15–7 Biotic and Abiotic Factors Affecting N_2 Fixation by Free-Living Soil Bacteria

Factor	Biotic or Abiotic	Effect on N_2 Fixation
Carbon/energy source	Biotic/abiotic	Lack of an abundant supply of available organic C is considered the principal limiting factor for N_2 fixation by free-living soil diazotrophs. (Recall the need for large quantities of ATP for nitrogenase activity.)
Oxygen	Abiotic	Nitrogenase is in most cases irreversibly damaged by exposure to O_2. N_2 fixation by aerobes is usually most vigorous when the O_2 level is much reduced, and it must generally be absent for the anaerobes and facultative anaerobes.
Combined nitrogen	Abiotic	Nitrogenase is strongly controlled by the presence of combined nitrogen (i.e., ammonium, nitrate, and organic N compounds such as amino acids) in soil.
Competition	Biotic	N_2-fixing bacteria must compete with all other soil microbes for carbon supplies, etc. It is generally agreed that diazotrophs constitute 1%–10% of the cultivable bacterial population.
Others	Biotic	Like all other soil bacteria, diazotrophs are subject to predation by protozoa and lysis by bacterial viruses (bacteriophages).
	Abiotic	pH, temperature, and trace element availability.

DINITROGEN FIXATION BY PLANT-ASSOCIATED BACTERIA: THE ASSOCIATIVE SYMBIOSES

The Nature of the Association

Examples abound of associations in which an otherwise free-living diazotroph associates with a plant. These more or less casual associations generally exhibit no morphological modification of the "host" or overt genetic interaction between the plant and the bacterium, such as those observed in the root-nodule symbioses described in Chapter 16. Instead, they tend to result from the bacterium establishing itself in an environment where a carbon source is made available, albeit somewhat inadvertently, by the plant. Diazotrophic bacteria have been found on leaf surfaces, the so-called phyllosphere, and in and around the roots, or the rhizosphere, of a wide range of plant species.

Diazotrophic bacteria have been observed in the intercellular spaces of the outer cell layers of roots as well as in the moribund cells of the outer layers and in vascular elements of some plants. The bacteria residing in the rhizosphere and at the root surface (Fig. 15–4 and Fig. 15–5) use root exudates, secretions, lysates, and

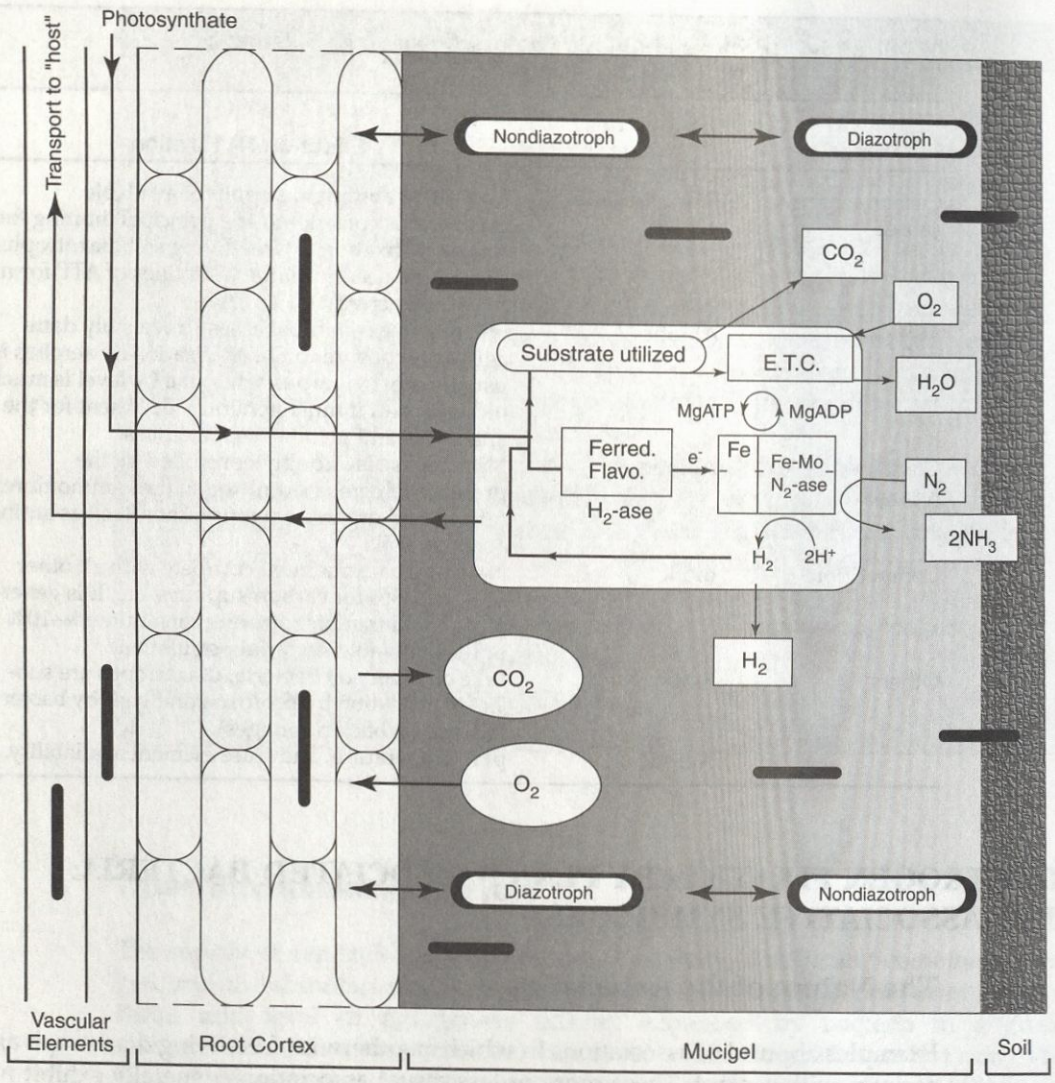
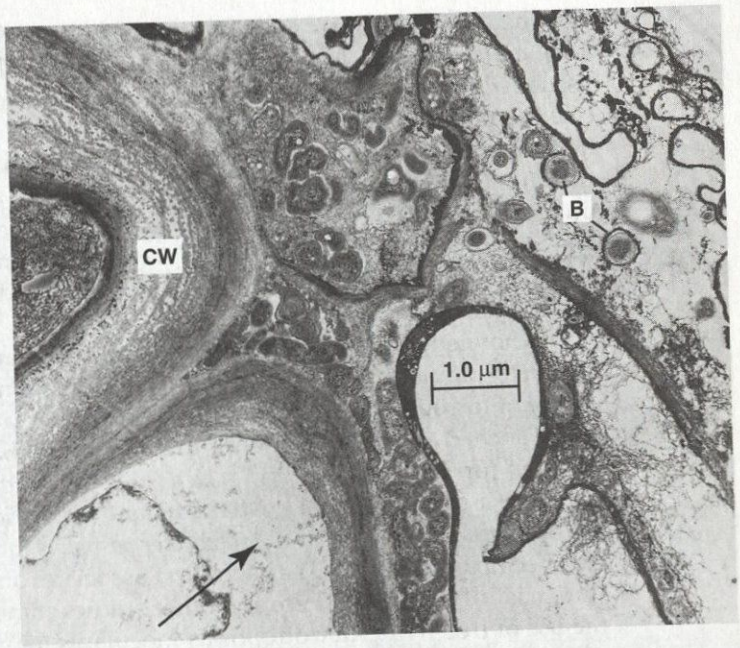
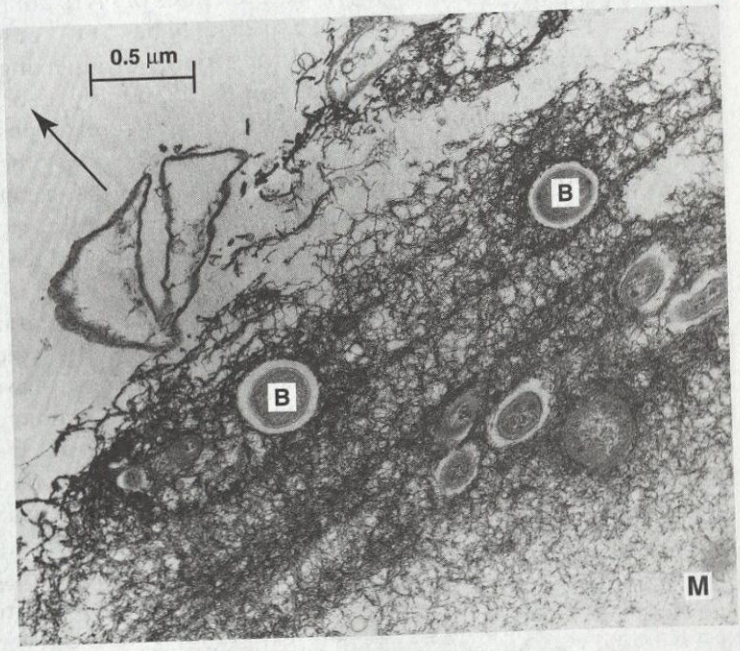


FIGURE 15–4
A model for root and rhizoplane-rhizosphere colonization by diazotrophic bacteria. The central reactions of the diazotroph are in the enlarged "cell" at center. Diazotrophs and nondiazotrophs (indicated by black bars) colonize the rhizosphere soil, the mucigel layer, the rhizoplane (root surface), and the outer cell layers of the root. In some instances, certain bacteria (for example, *Gluconacetobacter diazotrophicus*) can colonize the vascular elements. The bacteria grow at the expense of carbon sources (photosynthate, exudates, secretions, lysates, and sloughed cells) provided by the plant and forming a gradient of substrates external to the root (shaded area). Diazotrophs must compete for substrates with nondiazotrophs. Dinitrogen is fixed by bacteria in all of the locations shown and eventually becomes available to the plant or other microbes when the cells die and become mineralized. Interactions between diazotrophs and nondiazotrophs may be beneficial (e.g., a nondiazotroph lowering the oxygen concentration or immobilizing ammonium or nitrate) or detrimental (e.g., nondiazotrophs consuming carbon that otherwise might have been available to support N_2 fixation). Abbreviations: Ferred. = ferredoxin, Flavo. = flavodoxin, E.T.C. = electron-transport chain.



a.



b.

FIGURE 15–5
Photomicrographs of ultra-thin sections of mangrove roots viewed with a transmission electron microscope. (a) Junction between two root cells (note the multilayered cell wall [cw] material at left) and bacterial cells (B) in the mucilage and sloughed cell debris at the root surface. Numerous bacteria are in this material. The arrow points away from the central axis of the root. (b) Bacteria embedded in the mucilage. In this micrograph the fibrillar mucilage appears in layers probably because the plant root had periods of increased exudation of carbon. For reference orientation, these photos represent the immediate exterior of the intact root which could be encompassed in a zone such as that marked with the box in Figure 15–6 and enlarged graphically in Figure 15–4.

sloughed cells as a carbon source for growth and N_2 fixation. Some bacteria, such as *Gluconacetobacter* (formerly *Acetobacter) diazotrophicus*, may occupy internal root tissues of sugarcane, including the vascular elements. This and other **endophytic** (see Chapter 11) N_2-fixing bacteria are the subjects of renewed interests in N_2 fixation among nonleguminous plants. As a consequence of their activities, some dinitrogen is fixed and is eventually made available for plant growth. Bacteria that take up residence in the vascular elements gain the obvious advantage of being "first in line" for substrates and thereby reduce the problem of competition from nondiazotrophs (see Fig. 15–4). It is widely accepted that diazotrophic bacteria can be active colonizers of plant roots.

Knowledge that diazotrophic bacteria are part of the root and rhizosphere bacterial population has prompted interest in using the bacteria as an alternate source of nitrogen for crop growth. In the mid to late 1970s considerable research was launched as a result of reports of diazotrophic bacteria fixing large amounts of nitrogen (up to 90 kg ha^{-1} yr^{-1}) in association with the roots of tropical grasses, such as *Paspalum notatum* cv. batatais and *Digitaria* species. The association between *Paspalum notatum* and *Azotobacter paspali* is interesting because of the apparent specificity involved. The bacterium associates only with the tetraploid cultivar "batatais" of the grass. These and other studies stimulated much more research on the use of free-living diazotrophs as inoculants for crop plants. Further aspects of the use of these bacteria as inoculants are discussed in Box 15–3.

Today, the widely accepted rates of N_2 fixation by root-associated bacteria are probably in the range of 5 to 25 kg N ha^{-1} yr^{-1} or growing season. Compare these rates with those reported for a range of N_2-fixing plant-microbe associations in Table 15–8. These low rates of N_2 fixation by associative bacteria are probably insignificant for production of the major cereal crops; however, they are significant for range and prairie grasses where the only other nitrogen inputs are from atmospheric deposition and possibly from the excretion products of animals. They may also prove yet to be significant in the culture of low-maintenance grasses for amenity planting and roadside vegetation, and they may be important for growth of tropical plants other than grasses.

Why are the rates of N_2 fixation by root- and rhizosphere-colonizing bacteria so low? The answer lies in the requirements of the typical nitrogenase system. The enzyme complex is irreversibly harmed by exposure to oxygen and is stringently regulated by the level of combined nitrogen (e.g., ammonium and nitrate) in the vicinity of the cell. Also large amounts of energy—and therefore large amounts of substrates—are needed to reduce dinitrogen. Next, consider the conditions that might be encountered around the roots of a plant growing in a normally well-aerated (i.e., not water-logged) soil. In the associative grass-bacteria systems the diazotrophic bacteria live on or in the root or in the rhizosphere soil immediately surrounding the root. They are encouraged there by the liberation of substrates from the "host" root (Fig. 15–4).

The oxygen concentration around roots can vary considerably, but in general it is high enough that some limitation on N_2 fixation would result from the effect of molecular oxygen on nitrogenase. A secondary effect of oxygen is that the diazotrophs would respire more carbon to protect the nitrogenase and thereby reduce the efficiency of N_2 fixation. In contrast, the formation of microsites with low pO_2

BOX 15–3

Does Inoculation with Free-Living Diazotrophs Benefit Crop Production?

Among the most widely studied of the associative symbioses is the association between *Azospirillum* and roots of numerous grass species, including most of the important cereal crops. The subject has been thoroughly reviewed by Okon (1994). We should point out that the idea of exploiting free-living diazotrophs for crop production is not new. Throughout the 1950s and 1960s, the Russians treated vast acreages of wheat with *Azotobacter* in a formulation called "azotobacterin" in hopes of increasing yields without applications of fertilizer nitrogen. This practice met with little success. A small percentage of the experiments showed increased growth due to inoculation and when increases were observed, they were on the order of 10% or just barely detectable.

Okon and Labandera (1994) summarized the results of 20 years of field inoculation experiments with the bacterium *Azospirillum*. They concluded that the majority of the experiments (60 to 70%) led to statistically significant yield responses on the order of 5 to 30%. They further concluded that successful experiments "appeared to be those in which researchers paid special attention to the optimal numbers of cells of *Azospirillum* in the inoculant, using inoculation methods where the optimal number of cells remained viable and available to colonize the roots (p. 1597)."

Although these results are encouraging for the use of bacterial inoculants to increase or maintain crop yields, we must point out that the scientific literature tends to be skewed by the traditional bias against publishing papers reporting negative results. Thus, many experiments where no inoculation responses were observed have never been published. Another important point to be learned from the large body of work dealing with inoculation of plants with *Azospirillum* is that it is now widely accepted that increases in plant growth due to inoculation with the bacterium are in all likelihood not due to increased N_2 fixation. Rather, they may be attributed to the production of plant growth-promoting hormones (e.g., auxins, giberellins, and cytokinins) or to a variety of plant physiological responses. For example, inoculated plants are reported to be more effective in taking up inorganic nutrients, and it has been reported that the bacteria applied as inoculants elicit changes in the permeability of the membranes of root cells. *Azospirillum*, then, not only is an N_2-fixing associate of the roots of many plant species, but also has the attributes of other so-called **plant-growth-promoting rhizobacteria** (PGPR, Chapter 11 and 22). Today researchers continue to look for microbes that exert beneficial effects on plants. Whether we will see the development of highly effective inoculants for agricultural crops remains a "work in progress." In the meantime, consumers should shop with caution when it comes to buying microbial products.

TABLE 15–8 Estimated Average Rates of Biological N_2 Fixation for Specific Organisms and Associations

Organism or System	Dinitrogen Fixed ($kg\ ha^{-1}\ yr^{-1}$)
Free-living microorganisms	
Cyanobacteria ("blue-green algae")	25
Azotobacter	0.3
Clostridium pasteurianum	0.1–0.5
Grass-bacteria associative symbioses	5–25
Plant-cyanobacterial associations	
Gunnera	12–21
Azolla	313
Lichens	39–84
Legumes	
Soybeans (*Glycine* max L. Merr.)	57–94
Cowpeas (*Vigna, Lespedeza, Phaseolus*, and others)	84
Clover (*Trifolium hybridum* L.)	104–160
Alfalfa (*Medicago sativa* L.)	128–600
Lupines (*Lupinus* sp.)	150–169
Nodulated nonlegumes	
Alnus (alders, e.g. red and black alders)	40–300
Hippophae (sea buckthorn)	2–179
Ceanothus (snow brush, New Jersey tea, California lilac)	60
Coriaria ("tutu" in New Zealand)	60–150
Casuarina (Australian pine)	58

Adapted from Stevenson (1982). Used with permission.

(low molecular oxygen concentrations) results from the consumption of oxygen by root and microbial respiration so that some diazotrophs might actually benefit from the reduced pO_2. Alexander and Zuberer (1989a) showed that nitrogenase activity associated with roots of corn and sorghum was greatest at pO_2 ranging from 1.3 to 2.1% (1.3 to 2.1 kPa), and markedly lowered as the pO_2 approached 5%. They also demonstrated that plants whose roots were exposed to $^{15}N_2$ at the low oxygen concentration contained more than 200 times as much ^{15}N as plants whose roots were exposed to air (Alexander and Zuberer, 1989b). Thus, the inhibition of nitrogenase activity by oxygen is reduced as the oxygen content of the environment decreases, but even at values approaching 5% the activity is nearly shut down.

Because nitrogenase activity is also closely regulated by combined nitrogen, the bacteria only fix dinitrogen when it is absolutely necessary. Thus, nitrogen levels in the soil solution around roots of fertilized crop plants may be sufficient to cause limitations on root-associated N_2 fixation. Dinitrogen fixation would be expected to occur only in areas where plant and microbial uptake by nondiazotrophs depleted the available supply of soil nitrogen to levels sufficient to derepress nitrogenase synthesis. This may be likely in grasslands where dense rooting and microbial colonization of roots consistently keep the supply of available nitrogen at very low levels, but in highly fertilized soils the prospects for high rates of N_2 fixation are limited.

Perhaps the most significant constraint on root- and rhizosphere-associated diazotrophs is lack of an adequate supply of readily available carbon sources coupled with the extremely high competition for any carbon liberated from the root. The rhizosphere is not populated exclusively with diazotrophic bacteria. In fact, they comprise only 1% to 10% of the recoverable rhizosphere population. Competition among these organisms for the limited supply of available carbon can place severe restrictions on N_2 fixation in the root zone. However, if the diazotroph can become established inside the root as an endophyte, the competition for substrates is lessened, particularly for cells in the vascular elements of the plant (see Box 15–4 on N_2 fixation in sugarcane). Many studies have shown that N_2 fixation in the rhizosphere is carbon-limited. Roots exhibiting little or no acetylene reduction by associative bacteria show high rates shortly after the addition of available carbon sources (e.g., sugars, organic acids) to the medium surrounding the root system. Marked stimulation of N_2 fixation usually occurs upon addition of readily available carbon sources to soil. The addition of the ready supply of carbon and the resulting immobilization of combined soil nitrogen make conditions favorable for high rates of N_2 fixation. Even incorporation of straw into soil can support some N_2 fixation.

BOX 15–4

Dinitrogen-Fixing Rice and Sugarcane?

Within the last three decades, a great deal of effort has been expended in looking for N_2-fixing plant-bacteria associations capable of providing agriculturally significant amounts of fixed nitrogen for crop production. The optimistic reports of the early 1970s suggesting high rates of N_2 fixation associated with roots of grasses were soon tempered by numerous reports that failed to corroborate the earlier findings. However, from this large body of work, scientists have been able to learn much about the nature of the associations between roots of grasses and a large variety of free-living, N_2-fixing bacteria (diazotrophs). What they learned is that there appear to be some systems where free-living diazotrophs can be of significant benefit to their "host" plants.

Two crops that appear to derive substantial benefit from their diazotrophic associates are rice grown under flooded conditions and sugarcane. Interestingly, both of these crops have been observed to be able to produce appreciable yields in the absence of added fertilizers. The ability of plants to grow well without exogenous fertilizers raises the suspicion that these plants might somehow be benefiting from N_2-fixing bacteria.

Consider the case for N_2 fixation in wetland (paddied) rice culture. Rice is the major staple crop for two-thirds of the world's people. For centuries in Asian countries, rice has been cultured without the use of fertilizers other than small inputs of human and animal manures, yet the crop bears good

BOX 15–4 *(continued)*

yields. Scientists suspected for a long time that N_2-fixing bacteria might somehow be involved in providing fixed N for the rice plant. As it turns out, because of the tolerance of rice plants for growth in flooded, often anoxic soils, they derive benefit from a variety of N_2-fixing microbes including free-living heterotrophic bacteria, N_2-fixing cyanobacteria, phototrophic bacteria, and a symbiotic partnership between the floating aquatic fern, *Azolla*, and the heterocystous cyanobacterium, *Anabaena*. The flooded conditions of the rice paddy overcome some of the environmental limitations on N_2-fixing bacteria and cyanobacteria. First, the floodwater is a much better environment for growth of algae and cyanobacteria than is an upland "dry" soil. Second, bacteria in the mud and associated with roots of rice plants find the low-oxygen environment protective of their nitrogenase enzyme. Thus the oxygen poisoning of nitrogenase is at least partially alleviated by the naturally reduced oxygen status of the muds and floodwater. Third, before the rice plants grow tall enough to shade the paddy surface, considerable light is available for growth of the diazotrophic photosynthetic bacteria and cyanobacteria. It has been estimated that rice may gain from 30 – 50 kg N ha^{-1} per growing season through the combined activities of these free-living microbes. The *Azolla-Anabaena* symbiosis (discussed further in the next chapter) can provide from 150–300 kg N ha^{-1} per growing season.

Though sugarcane is not grown in flooded soils, it too has been observed to yield well with seemingly little input of fertilizer nitrogen. During the 1980s Dr. Johanna Döbereiner and others working in Brazil discovered that some cultivars of sugarcane contained a diazotrophic bacterium that they named *Acetobacter diazotrophicus* (recently renamed *Gluconacetobacter diazotrophicus*). The organism can be isolated from the internal tissues of roots, stems, and leaves. It is unique in that it will grow in a 30% sucrose solution, will fix N_2 optimally at pH 5.5 (and less well at values approaching pH 3), and its N_2-fixing capability seems to be more tolerant of oxygen than other free-living bacteria. Researchers in Brazil and elsewhere have since confirmed the unique association between this bacterium and sugarcane. The bacterium has been observed in the intercellular spaces as well as in the xylem elements of sugarcane stems and it may achieve numbers on the order of one to ten million cells g^{-1} of these tissues and one to ten thousand cells ml^{-1} of fluid in the intercellular spaces (Dong et al., 1994; James, et al., 1994). Occupying such a unique location, the organism is poised to intercept a large supply of sugar to drive N_2 fixation and it is free of the competition from other bacteria that organisms in the rhizosphere must deal with! It has been estimated that some sugarcane cultivars might derive as much as 100–150 kg N ha^{-1} from this unique association. Time will tell what the real significance of this bacterium is in the commercial production of sugarcane and perhaps other crops.

To put the limitation of carbon supply on N_2 fixation into perspective, consider a statement from Giller and Day (1985) about estimating maximal possible rates of N_2 fixation for a given amount of root carbon.

"If one assumes that:

- the efficiency of conversion of root carbon is 10 g C g^{-1} N fixed,
- N_2-fixing bacteria comprise 10% of the total rhizosphere population (a high estimate) and acquire organic carbon in proportion to their numbers,
- all of the carbon translocated below ground is equally available for use by all bacteria,

then:

- if 1500 kg C ha^{-1} are translocated below ground as estimated for a wheat crop, the maximum potential N_2 fixation is 15 kg N ha^{-1}" (p. 133).

Recall that most estimates of N_2 fixation in grasslands range from 5 to 25 kg N ha^{-1}. It is clear that conditions are conducive for N_2 fixation in the rhizosphere, at least part of the time during the growing season. It is also likely that the integration of biotic and abiotic factors provides favorable microsites for N_2 fixation to occur at relatively high rates for short periods or at low rates for longer periods.

The factors (biotic and abiotic) affecting N_2 fixation by bacteria associated with belowground plant parts are summarized in Figure 15–6 and Table 15–7. Remember that the bacteria are dependent on the "host" plant for provision of carbon sources; thus anything that affects the photosynthesis of the plant indirectly affects the bacteria associated with the roots or other belowground structures such as nodules. Also important are the combined effects of the soil variables (moisture, aeration, pH, temperature, and nitrogen content) on plant and microbial growth. By studying the simple models presented in Figures 15–4 and 15–6, we can develop an appreciation of how all factors must come together at appropriate levels to allow for expression of N_2 fixation by bacteria in the roots or rhizospheres of nonleguminous plants like the grasses. We can also gain an appreciation of the many factors that are "taken care of" by the host plants in the exquisite root nodule symbioses discussed in Chapter 16.

METHODS FOR MEASURING DINITROGEN FIXATION

The agronomic and ecological importance of biological N_2 fixation has driven intensive study of the process for the past century. Early studies of biological N_2 fixation were limited by the lack of techniques sensitive enough to measure nitrogen gains in plants or to measure the activity of the enzyme complex itself. As developments in electronics have evolved rapidly, so too has the development of sophisticated instrumentation for measurement of biological processes, including N_2 fixation. Here the methods routinely used for measurement of N_2 fixation or

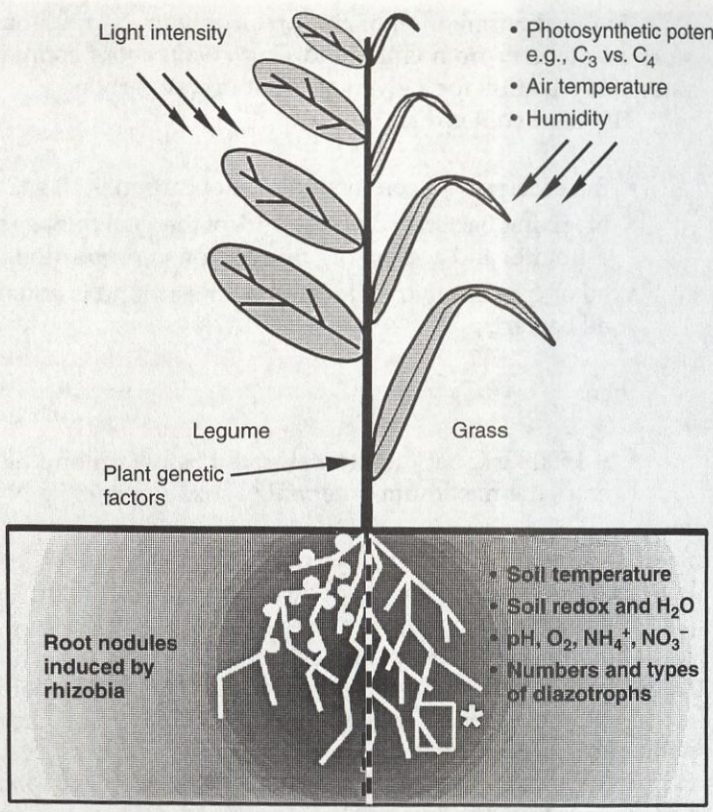


FIGURE 15–6
Main factors affecting nitrogenase activity belowground include: light intensity, potential of the photosynthetic apparatus (e.g., C_3 vs. C_4 plants), air temperature (19°C min.), air water deficit (affects stomatal activity and water balance), soil temperature, soil redox and water content (18% water content considered an approximate minimum), other soil factors (e.g., pH, O_2 and N content), plant genetic factors, and the nature and numbers of the diazotrophs associated with roots. The starred box is represented in Figure 15–4.

nitrogenase activity will be introduced. Detailed reviews of the subject are available elsewhere (Bergerson, 1980; Sprent and Sprent, 1990; Weaver and Danso, 1994).

Measurement of Dinitrogen Fixation by the Nitrogen Difference Method

It is possible to estimate N_2 fixation by comparing the yields and nitrogen content of plants grown with and without N_2-fixing bacteria. One example is comparing the yields of nodulated and nonnodulated legumes grown under similar conditions of soil nitrogen. The nitrogen in the nonfixing plant is a measure of the nitrogen acquired from soil and is subtracted from the nitrogen of the fixing plant; the difference is attributed to N_2 fixation. Weaver and Danso (1994) indicate that the

sensitivity of the method is such that an increase of about 20 kg N ha^{-1} would be required for detection of significant differences. The method is limited to the study of systems where N_2 fixation rates are high.

Measurement of Dinitrogen Fixation by the Acetylene Reduction Assay

The ability of the nitrogenase complex to reduce acetylene (C_2H_2) to ethylene (C_2H_4) forms the basis for the acetylene reduction assay (see Burris, 1974, for a description of development of the method). The sensitivity of this method, based on gas chromatography, allowed detection of very low levels of nitrogenase activity. In fact, the sensitivity of the acetylene reduction assay facilitated attempts to measure N_2 fixation by grasses.

The reaction for the reduction of dinitrogen to ammonia is:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

The conversion of acetylene to ethylene occurs by the following reaction:

$$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4$$

The reduction of dinitrogen (including reductant used in H_2 evolution) requires eight electrons, whereas the reduction of acetylene requires two electrons. Thus, reduction of four molecules of acetylene is equivalent to reduction of one molecule of N_2; that is, the ratio of acetylene reduced to nitrogen fixed is 4/1. In practice, this ratio has been shown to vary anywhere from 1.5/1 to 25/1, depending on the system being measured. Most investigators assume a ratio of 3/1 to 4/1.

In the acetylene reduction assay, the system to be measured (whole plants, isolated roots, soil cores, or bacterial cultures) is exposed to an atmosphere containing 11% acetylene and incubated under appropriate conditions. The concentration of acetylene is such that the nitrogenase complex is saturated with this substrate and dinitrogen, the normal substrate (Fig. 15–2), is no longer reduced. Samples of the gas phase are periodically removed and injected into the gas chromatograph for quantification of ethylene production from acetylene.

The acetylene reduction assay is not without limitations—careful attention must be paid to these limitations to avoid gross overestimations of N_2 fixation—and the method is still a point of some contention among researchers. Pitfalls of the acetylene reduction assay for measurement of associative N_2 fixation include (Giller, 1987):

- microbial production of ethylene which is not from acetylene,
- oxidation of ethylene by other soil bacteria,
- nitrogen starvation during prolonged incubations due to inhibition of nitrogenase by acetylene, and
- failure to calibrate the acetylene reduction vs. dinitrogen fixation ratio appropriately.

Measurement of Dinitrogen Fixation by Stable Isotope (^{15}N) Methods

The most definitive measurements of biological N_2 fixation make use of the stable, heavy isotope ^{15}N and require access to a mass spectrometer. The availability of these instruments is not as major a limitation as it once was but ^{15}N methods are still expensive, because of both the high costs of the ^{15}N-labeled nitrogen sources and the cost of analysis.

The principal methods involved in studies using ^{15}N to measure N_2 fixation are:

- measurement of the incorporation of $^{15}N_2$ (labeled dinitrogen) into plant or microbial cells,
- isotope dilution methods in which the content of ^{15}N in plant tissue is measured and the ratio of ^{15}N to ^{14}N is calculated, and
- natural abundance of ^{15}N in soils or plants.

The first method is straightforward. Samples are exposed to an atmosphere of about 10% $^{15}N_2$, usually in a balance of argon or helium (with sufficient oxygen and carbon dioxide) to eliminate competition from $^{14}N_2$. Control plants not exposed to $^{15}N_2$ are included to measure background ^{15}N. Following incubation, the samples (cells or ground plant materials) are digested and the ^{15}N content of the materials is determined using a mass spectrometer. Detection of ^{15}N in tissues or cells provides definitive proof of N_2 fixation and allows very accurate quantification of the amount of dinitrogen fixed.

The *isotope dilution* and *natural abundance* methods rely on the fact that in nature the ratio of $^{15}N/^{14}N$ is remarkably constant. ^{15}N and ^{14}N constitute about 0.3663% and 99.6337% of the nitrogen in the atmosphere, respectively. A substance containing more ^{15}N is said to be ^{15}N-enriched. Such materials can be exploited to measure N_2 fixation in the field. In practice, the soil is fertilized to a low level with the ^{15}N-enriched fertilizer, and the plants are grown to maturity or a suitable stage for harvest. The ^{15}N content in the tissues is analyzed and the nitrogen derived from the atmosphere can be calculated. The fixing plant will derive part of its nitrogen from the atmosphere and will contain more ^{14}N than the soil nitrogen, thus the ^{15}N in the fixing plant is "diluted" with the ^{14}N from atmospheric dinitrogen.

The ^{15}N natural abundance method is based on the same principle as the isotope dilution method. Natural materials contain nitrogen that is naturally enriched with ^{15}N because of isotope discrimination during biological transformations of nitrogen in soil. Therefore, plants containing less ^{15}N than the natural abundance can be suspected of supporting N_2 fixation. The assumptions and formulae for calculating the amount of nitrogen fixed and the rationale for the calculations are given by Weaver and Danso (1994).

SUMMARY

In this chapter we introduced the fundamentals of the process of biological N_2 fixation and the nature of the enzyme complex, nitrogenase, which mediates the conversion of dinitrogen to ammonia. The process is unique in that only prokaryotic microbes, as far as we know, possess this enzyme system. Just as it is expensive to fix dinitrogen industrially, it is also expensive biologically for the microbes that fix dinitrogen. Therefore, high rates of biological N_2 fixation occur only when a ready supply of energy—light for phototrophs and organic carbon for organotrophs—is available. For this reason, the organotrophic N_2-fixing microbes are usually found in association with plants or in soils receiving organic carbon additions.

Despite the cumbersome nature of the nitrogenase enzyme complex, about two-thirds of the global supply of fixed nitrogen is produced by biological N_2 fixation, mostly through the legume-rhizobia symbiosis discussed in Chapter 16. Though the contributions of fixed nitrogen from the nonsymbiotic diazotrophs are generally low (5 to 25 kg N ha^{-1} yr^{-1}), they are significant in areas where no other source of nitrogen is available. The associative symbioses between grasses and free-living diazotrophs continue to receive attention not only for their interesting biology but with the objective of enhancing such associations for practical use in sustaining agricultural and natural ecosystems.

It is difficult to foresee what breakthroughs will spring from continued investigations of nitrogenase and the microbes that possess it. Imagine if chemists, biochemists, and microbiologists could someday mimic the action of nitrogenase in huge tanks to fix dinitrogen without the high costs of the fossil fuel currently needed. Imagine if molecular biologists could someday get N_2-fixing root nodules on nonleguminous plants, such as wheat, rice, and corn. Then a whole new set of questions would arise. For example, can these plants provide the carbon necessary to support N_2-fixing nodules and still produce abundant grain? These are dreams perhaps, but it is reasonably certain that N_2 fixation has a strong future in agricultural research and production and in the maintenance of our relatively unmanaged ecosystems such as forests and grasslands.

CITED REFERENCES

Alexander, D. B., and D. A. Zuberer. 1989a. Impact of soil environmental factors on rates of N_2 fixation associated with intact maize and sorghum plants (pp. 273–285). In F. A. Skinner, R. M. Boddey, and I. Fendrik (Eds.), *Nitrogen fixation with non-legumes*. Kluwer Academic Press, Dordrecht, The Netherlands.

Alexander, D. B., and D. A. Zuberer. 1989b. $^{15}N_2$ fixation by bacteria associated with maize roots at a low partial O_2 pressure. *Appl. Environ. Microbiol.* 55: 1748–1753.

Bergerson, 1980. *Methods for evaluating biological nitrogen fixation.* John Wiley and Sons, New York, NY.

Bishop, P. E, and R. Premakumar. 1992. Alternative nitrogen fixation systems (pp. 736–762). In G. Stacey, R. H. Burris, and H. J. Evans (Eds.), *Biological nitrogen fixation*. Chapman and Hall, New York.

Burns, R. C., and R. W. F. Hardy. 1975. *Nitrogen fixation in bacteria and higher plants.* Springer-Verlag, New York.

Burris, R. H. 1974. Methodology (pp. 10–33). In A. Quispel (Ed.), *The biology of nitrogen fixation*. American Elsevier Publishing Co., New York.

Burris, R. H., and G. P. Roberts. 1993. Biological nitrogen fixation. *Annu. Rev. Nutr.* 13: 317–335.

Dixon, R. O. D., and C. T. Wheeler. 1986. *Nitrogen fixation in plants*. Chapman and Hall, New York.

Dong, Z., M. J. Canny, M. E. McCully, M. R. Roberedo, C. F. Cabadilla, E. Ortega, and R. Rodés. 1994. A nitrogen-fixing endophyte of sugarcane stems. A new role for the apoplast. *Plant Physiol.* 105: 1139–1147.

Evans, H. J., and R. H. Burris. 1992. Highlights in biological nitrogen fixation during the last 50 years (pp. 1–42). In G. Stacey, R. H. Burris, and H. J. Evans (Eds.), *Biological nitrogen fixation*. Chapman and Hall, New York.

Giller, K. E. 1987. Use and abuse of the acetylene reduction assay for measurement of "associative" nitrogen fixation. *Soil Biol. Biochem.* 19: 783–784.

Giller, K. E., and J. M. Day. 1985. Nitrogen fixation in the rhizosphere: Significance in natural and agricultural systems (pp. 127–147). In A. H. Fitter (Ed.), *Ecological interactions in soil*. Blackwell Scientific Publications, Oxford, England.

Hauck, R. D. 1985. Agronomic and technological approaches to improving the efficiency of nitrogen use by crop plants (pp. 317–326). In K. A. Malik, S. H. M. Naqvi, and M. I. H. Aleem (Eds.), *Nitrogen and the environment*. Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan.

Hill, S. 1992. Physiology of nitrogen fixation in free-living heterotrophs (pp. 87–134). In G. Stacey, R. H. Burris, and H. J. Evans (Eds.), *Biological nitrogen fixation*. Chapman and Hall, New York.

James, E. K., V. M. Reis, F. L. Olivares, J. I. Baldani, and J. Döbereiner. 1994. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *J. Expt. Bot.* 45: 756–766.

Okon, Y., J. P. Houchins, S. L. Albrecht, and R. H. Burris. 1977. Growth of *Spirillum lipoferum* at constant partial pressures of oxygen and the properties of its nitrogenase in cell-free extracts. *J. Gen. Microbiol.* 98: 87–93.

Okon, Y., and C. Labandera. 1994. Agronomic application of *Azospirillum:* An evaluation of 20 years worldwide field inoculation. *Soil Biol. Biochem.* 26: 1591–1601.

Okon, Y. 1994. Azospirillum/*plant associations*. CRC Press, Boca Raton, Fla.

Postgate, J. R. 1998. *Nitrogen fixation* (3rd ed.). Edward Arnold, London.

Postgate, J. 1994. *The outer reaches of life*. Cambridge University Press, Cambridge, England.

Ribbe, M., D. Gadkari, and O. Meyer. 1997. N_2 fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide

oxidoreductase that couples N_2 reduction to the oxidation of superoxide produced from O_2 by a molybdenum CO dehydrogenase. *J. Bio. Chem.* 272: 26627–26633.

Sprent, J. I., and P. Sprent. 1990. *Nitrogen fixing organisms. Pure and applied aspects.* Chapman and Hall, New York.

Stevenson, F. J. 1982. Origin and distribution of nitrogen in soil (pp. 1–42). In F. J. Stevenson (Ed.), *Nitrogen in agricultural soils.* Agronomy No. 22, American Society of Agronomy, Madison, Wis.

Waggoner, P. E. 1994. *How much land can ten billion people spare for nature?* Council on Agricultural Science and Technology, Report 121, Ames, Ia.

Weaver, R. W., and S. K. A. Danso. 1994. Dinitrogen fixation (pp. 1019–1045). In R. W. Weaver, S. Angle, P. Bottomley, D. Bedzdicek, S. Smith, A. Tabatabai, and A. Wollum (Eds.), *Methods of soil analysis, Part 2. Microbiological and biochemical properties.* Soil Science Society of America, Book Series, No. 5. Madison, Wis.

Young, J. P. W. 1992. Phylogenetic classification of nitrogen-fixing organisms (pp. 43–86). In G. Stacey, R. H. Burris and H. J. Evans (Eds.), *Biological nitrogen fixation.* Chapman and Hall, New York.

GENERAL REFERENCES

Döbereiner, J., and F. O. Pedrosa. 1987. *Nitrogen-fixing bacteria in nonleguminous crop plants.* Science Tech Publishers, Madison, Wis.

Elmerich, C., W. Zimmer, and C. Vielle. 1992. Associative nitrogen-fixing bacteria (pp. 212–258). In G. Stacey, R. H. Burris, and H. J. Evans (Eds.), *Biological nitrogen fixation.* Chapman and Hall, New York.

Schlesinger, W. H. 1997. *Biogeochemistry. An analysis of global change* (2nd ed.). Academic Press, New York.

Zuberer, D. A. 1990. Soil and rhizosphere aspects of N_2-fixing plant-microbe associations (pp. 317–353). In J. M. Lynch (Ed.), *The rhizosphere.* John Wiley and Sons, New York.

Zuberer, D. A. 2001. Nitrogen fixation in soils-free-living microbes. In G. Bitton (Ed.), *Encyclopedia of environmental microbiology.* John Wiley & Sons, New York.

STUDY QUESTIONS

1. Discuss the significance of biological N_2 fixation in terms of its relative contribution to the world's supply of fixed nitrogen.
2. Write the biochemical reaction for the process of biological N_2 fixation.
3. What kinds of microorganisms are capable of carrying out biological N_2 fixation? Why are these microbes called "diazotrophs"?