Chapter 2
Resource Acquisition

To grow, both plants and bacteria utilize nutrients that are mainly acquired from the soil environment. In addition to small amounts of a number of different metals, plants and bacteria require fixed nitrogen, iron, and phosphorus. In this chapter, the mechanisms and genes involved in PGPB and plant resource acquisition are discussed in some detail. In fact, one of the major benefits that PGPB provide to their plant partners is that they facilitate the plant's acquisition of resources from the soil.

2.1 Nitrogen Fixation

One of the major nutrients necessary for the growth of all living organisms including plants and bacteria is nitrogen. Despite nitrogen's abundance in the earth's atmosphere, i.e., ~80 %, it must first be reduced to ammonia before it can be metabolized by plants to become an integral component of proteins, nucleic acids, and other biological molecules. This conversion requires a high input of energy because the triple bond of N_2 is extremely stable. Consequently, contemporary agriculture, especially in more developed countries, relies heavily on the use of nitrogen fertilizers derived at the expense of petroleum. In fact, 1.3 tons of oil, or an equivalent amount of energy, are needed to fix 1 ton of ammonia, and more than 100 million tons of fixed nitrogen are needed annually to sustain global food production. Besides being costly, the production of chemical nitrogen fertilizers depletes non-renewable resources and poses human and environmental hazards. On the other hand, nature has ingeniously solved this problem through the process of biological nitrogen fixation. Nitrogen-fixing bacteria, or diazotrophs, annually produce about 60 % of the earth's newly fixed nitrogen. With the objective of reducing the world's dependency on energy intensive chemical

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fertilizers, over the past 30–40 years there has been a large body of research directed toward understanding and eventually utilizing biologically fixed nitrogen in agriculture.

A wide range of bacteria can fix nitrogen, and a number of them have potential as crop fertilizers; eukaryotes do not fix nitrogen. Diazotrophic bacteria include, but are not limited to, several genera of cyanobacteria; the symbiotic genera *Rhizobia*, *Sinorhizobia*, *Bradyrhizobia*, *Mesorhizobia,* and *Frankia*; rhizospheric; and endophytic genera *Azospirillum*, *Azotobacter*, *Klebsiella*, *Bacillus,* and *Pseudomonas*.

2.1.1 Cyanobacteria

Cyanobacteria, previously called blue-green algae, share some characteristics of both bacteria and plants. Although they share many characteristics with gram-negative bacteria, cyanobacteria contain chlorophyll a and phycobiliproteins (proteins that capture light energy which is passed on to chlorophylls during photosynthesis). Cyanobacteria are capable of both photosynthesis (fix atmospheric CO_2) and nitrogen fixation; however, while all cyanobacteria are photosynthetic, only some can fix nitrogen. A number of different studies have been reported on the use of dried cyanobacteria as a fertilizer to inoculate soils and aid fertility. Moreover, there are a large number of studies in which cyanobacteria have been added to rice paddies with the result that the nitrogen that they fix and release may be taken up and used by the rice plants. This strategy is typically quite effective as sufficient fixed nitrogen is often considered to be rice's major growth-limiting factor. Following seed germination and a short growth phase in a greenhouse, rice seedlings are generally transplanted to paddies where the roots and a portion of the plant are continuously submerged. It is estimated that globally there are more than 160 million hectares of land devoted to the cultivation of rice. Diazotrophic cyanobacteria may be added to the water in the paddy where they proliferate and release some of their fixed nitrogen into the water to be taken up by the rice plants. In addition to rice, in many topical and subtropical environments, other crops including vegetables, wheat, sorghum, corn, cotton, and sugarcane are also grown using cyanobacteria as a biofertilizer. Moreover, in some regions, the heterocystous cyanobacterium-*Anabaena azollae* is found within the leaf cavity of the fern Azolla with this partnership acting as a traditional biofertilizer.

Some cyanobacteria such as *Nostoc* spp. and *Anabaena* spp. consist of long chains of two different types of cells, vegetative cells, and heterocysts (Fig. 2.1). The vegetative cells, typically about 90–95 % of the total number of cells, are photosynthetic while the larger and thick-walled heterocysts are nitrogen fixing. The vegetative cells and the heterocysts exchange nutrients so that all of the cyanobacterial cells have sufficient levels of both nitrogen and newly fixed carbon necessary to support the growth of the organism. Nitrogen is typically transported as glutamine while carbon may be transported as sucrose.

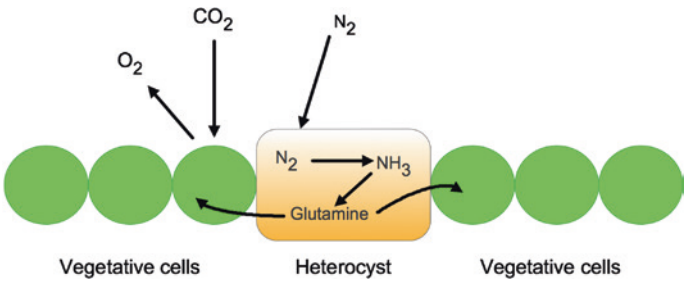

Fig. 2.1 Schematic representation of a portion of a chain of *Anabaena* cells showing photosynthetic vegetative cells and nitrogen-fixing heterocyst cells

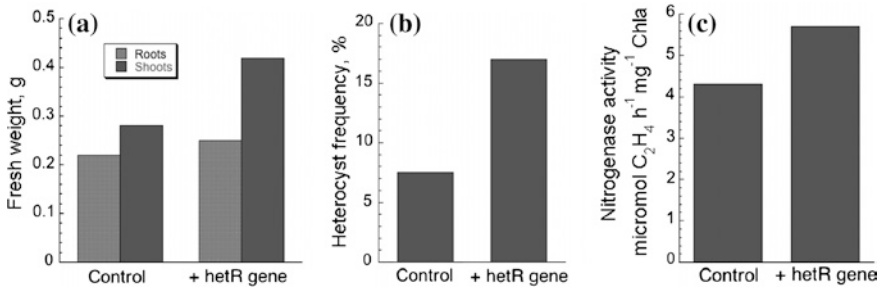

Fig. 2.2 Improvement of **a** fresh weight of root and shoot cells, **b** heterocyst frequency (i.e., the number of heterocysts divided by the total number of cells), and **c** the nitrogenase activity of control (wild-type) versus recombinant cells that overexpress the *hetR* gene in *Anabaena* sp. The data is from Appl. Environ. Microbiol. 77:395–399 (2011)

In one interesting experiment, scientists were able to genetically manipulate *Anabaena* sp. strain PCC7120 so that it contained an increased number of heterocysts and therefore fixed more nitrogen than usual. Recently, the *hetR* gene, which encodes a serine-type protease, has been identified as a master regulator of heterocyst differentiation. Thus, one group of researchers cloned the *Anabaena hetR* gene, expressed it under the control of a strong light-inducible *Anabaena* promoter, and integrated the entire construct into an intergenic region within the *Anabaena* genome, with the final construct containing two copies of the *hetR* gene—the original gene and the recombinant gene. The resulting high expression of an additional copy of the *hetR* gene elevated HetR protein expression and increased rice plant fresh weight and the amount of fixed nitrogen in the recombinant strain (Fig. 2.2). The ability of *Anabaena*/*Nostoc* strains to grow and be sustained photoautodiazotrophically (being both photosynthetic and nitrogen fixing) under tropical light, moisture, and temperature means that these strains can be used as nitrogen biofertilizers in flooded rice fields. This clever and simple strategy works well under laboratory conditions; however, it remains to be seen whether regulatory agencies in various countries will permit this recombinant cyanobacterial strain to be used in the open environment.

In addition to providing fixed nitrogen, cyanobacteria may also benefit crop plants by providing various growth-promoting substances such as gibberellins, auxins, vitamins, free amino acids, and various carbohydrates and sugars. Moreover, many cyanobacteria are able to solubilize inorganic phosphate and make it available for crop growth.

Given their ability to fix both carbon and nitrogen, there has recently been considerable interest in utilizing cyanobacteria as microbial cell factories as an alternative for bioenergy production. Some of the advantages of using cyanobacteria to synthesize various biofuels include the following (i) they grow relatively rapidly, (ii) under favorable weather conditions, they can be grown during the entire year, (iii) they can be cultivated in brackish water and on non-arable land, (iv) they can sometimes be grown on wastewater, (v) they do not require fertilizers, pesticides, or herbicides, and (vi) after the biofuel has been extracted, the residual biomass may sometimes be useful as a cattle feed. Unfortunately, to be cost-effective, the large-scale growth of cyanobacteria is often performed in open tanks that may be subject to contamination. While there are not as yet any commercial products that have been produced from either wild-type or by genetically engineered cyanobacteria, several products have been produced on a small scale by cyanobacteria under laboratory conditions including ethanol, isobutyraldehyde, isobutanol, 1-butanol, isoprene, hydrogen, fatty acids, and fatty alcohols.

2.1.2 Rhizobia

At the present time, ever-increasing amounts of *Rhizobia* are being utilized in conjunction with the growth of legume crops as an alternative to chemically fixed nitrogen. In addition, the extensive biochemical and molecular biological studies of symbiotic diazotrophs, such as rhizobia, have served as a conceptual starting point for understanding some of the mechanisms of growth promotion by other PGPB.

Rhizobia are gram-negative, flagellated, and rod shaped, and they form symbiotic relationships with legumes. Generally, each rhizobial species is specific for a limited number of plants and will not interact with plants other than its natural hosts (Table 2.1). In general, scientists isolate new strains of rhizobia by culturing the bacteria that are found within legume root nodules.

As part of their life cycle, rhizobial bacteria invade plant root cells and initiate a complex series of developmental changes that lead to the formation of a root nodule. Inside the root nodule, the bacteria proliferate and persist in a form called a bacteroid that has no cell wall (Fig. 2.3). The bacteria within the nodules fix atmospheric nitrogen by means of the enzyme nitrogenase. The structural and biochemical interactions between a symbiotic rhizobacterium and a host plant are quite complex and mutually beneficial (Fig. 2.4). Inside a nodule, nitrogenase is protected from the toxic effects of atmospheric oxygen in two ways. First, oxygen does not readily diffuse into the thick-walled nodule. Second, the oxygen content within a nodule is regulated by the protein leghemoglobin. The heme moiety of

Table 2.1 Plant specificities of some rhizobial species

Bacterial species	Host plant(s)
Azorhizobium caulinodans	West African legume (*Sesbania rostrata*)
Bradyrhizobium elkanii	Soybean (*Glycine max*), black-eyed pea (*Vigna unguiculata* subsp. *dekindtiana*), mung bean (*Vigna radiata*)
Bradyrhizobium japonicum	Soybean (*G. max)*
Mesorhizobium amorphae	Desert false indigo (*Amorpha fruticosa*)
Mesorhizobium ciceri	Chickpea (*Cicer arietinum*)
Mesorhizobium chacoense	White carob tree (*Prosopis alba*)
Mesorhizobium huakuii	Chinese milk vetch (*Astragalus sinicus*)
Mesorhizobium loti	Lotus (*Lotus japonicus*)
Mesorhizobium mediteraneum	Chickpea (*C. arietinum*)
Mesorhizobium tianshanense	7 Legume species
Rhizobium sp. strain NGR234	>100 Tropical legume species
Rhizobium etli	Kidney bean (*Phaseolus vulgaris*), mung bean (*V. radiata*)
R. etli bv. mimosae	Mimosa (*Mimosa affinis*)
Rhizobium galegae	Goat's rue (*Galega officinalis, Galega orientalis*)
Rhizobium gallicum	Common bean (*P. vulgaris)*
Rhizobium huautlense	Danglepod (*Sesbania herbacea*)
Rhizobium leguminosarum bv. phaseoli	Kidney bean, mung bean
R. leguminosarum bv. trifolii	Clover (*Trifolium* spp.)
R. leguminosarum bv. viciae	Pea (*Pisum sativum*)
Rhizobium sullae	Sweetvetch (*Hedysarum coronarium*)
Rhizobium tropici	Mimosoid trees (*Leucaena* spp.) and some tropical legume trees (*Macroptilium* spp.)
Sinorhizobium fredii	Soybean (*G. max*)
Sinorhizobium meliloti	Alfalfa (*Medicago sativa*)
Sinorhizobium morelense	White popinac (*Leucaena leucocephala*)

Abbreviation: *bv* biovar

this oxygen-binding protein is synthesized by the bacterium. On the other hand, the globin portion of the leghemoglobin molecule is encoded within the plant genome. The leghemoglobin imparts a red color to the inside of the nodule (in much the same way that mammalian hemoglobin is colored red). As a consequence of this symbiotic relationship, the plant provides the bacterium with photosynthetically fixed carbon (generally in the form of organic acids), which the bacterium requires for growth. In turn, nodule bacteria provide the plant with fixed nitrogen.

2.1.2.1 Nitrogenase

The interest in diazotrophs as biological fertilizers overlapped the development of techniques for gene isolation and manipulation, thereby providing the impetus for studying the biochemical and molecular biological aspects of nitrogen

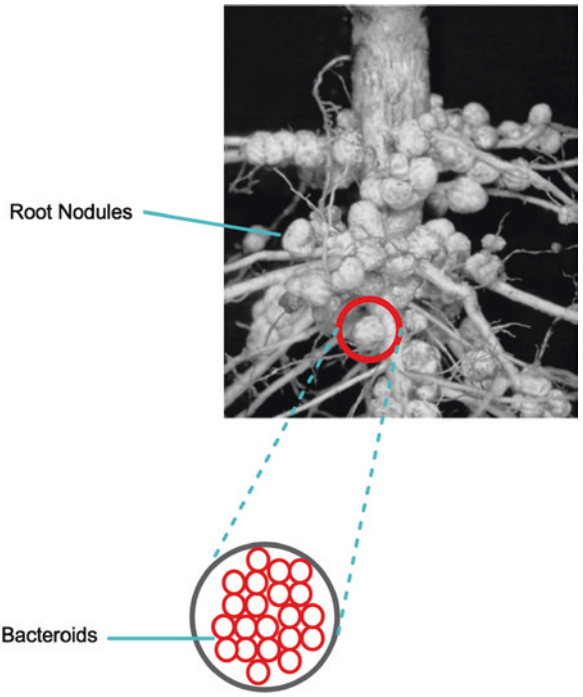

Fig. 2.3 Schematic representation of *red-colored* bacteroids within a plant root nodule

fixation. Initially, these studies were undertaken in the belief that they would lead to the development of improved nitrogen-fixing organisms that would enhance crop yields to a greater extent than the wild-type strains. Some researchers even went so far as to suggest that bacterial genes for nitrogen fixation might be introduced directly into plants to enable them to fix their own nitrogen. While these ideas have not been realized, a consequence of this research is that a detailed understanding of the process of nitrogen fixation has emerged. And with this understanding, the possibility of improving the nitrogen-fixing activity of some diazotrophs by genetic manipulation is a little closer to becoming a reality.

All known nitrogenases have two oxygen-sensitive components. Component I is a complex of two identical α-protein sub-units (approximately 50,000 daltons each), two identical β-protein sub-units (approximately 60,000 daltons each), 24 molecules of iron, 2 molecules of molybdenum, and an iron–molybdenum cofactor, sometimes called FeMoCo (Fig. 2.5). Component II has two α-protein sub-units (approximately 32,000 daltons each), which are not the same as the α-protein sub-units of component I, and a number of associated iron molecules. The catalysis of nitrogen to ammonium ion requires the combination of components I and II, a complex of magnesium and ATP, and a source of reducing equivalents (Fig. 2.6). In addition to fixing nitrogen, the nitrogenase can reduce the gas acetylene to ethylene. The measurement by gas chromatography of the increase in ethylene production (or the decrease in the acetylene level) as a function of time provides a convenient (and

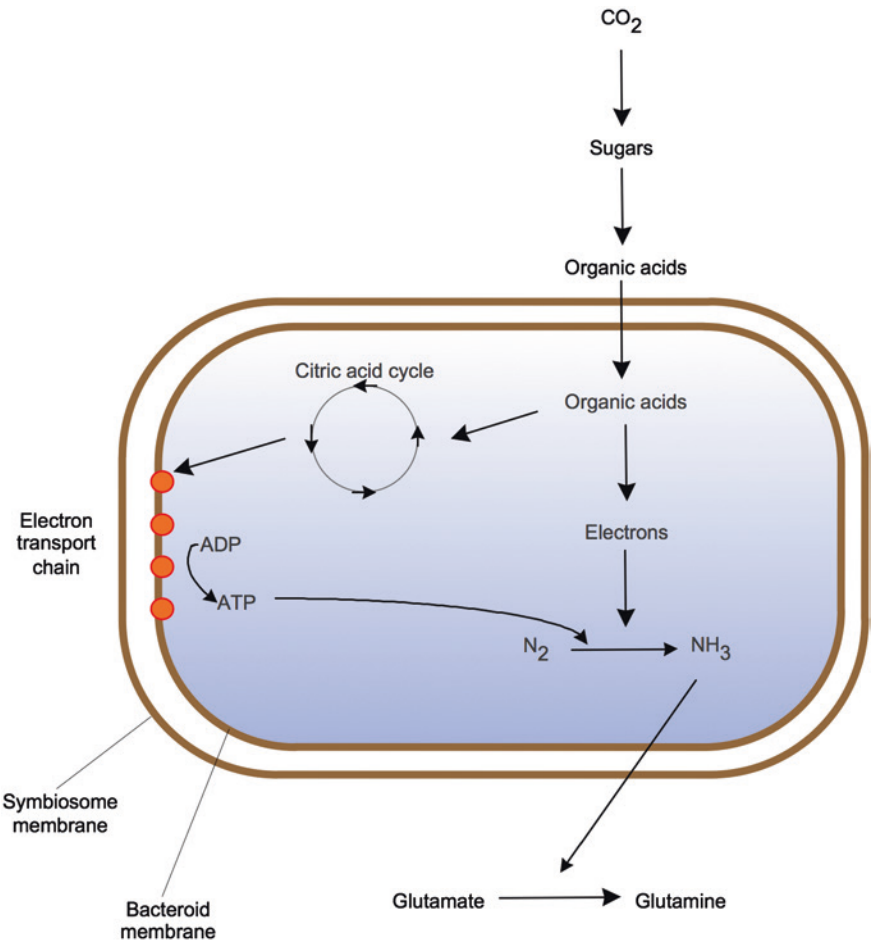

Fig. 2.4 Bacteroid metabolism. Dicarboxylic acids that were synthesized as a consequence of photosynthesis are taken up by the bacteroid. These acids are metabolized by the enzymes from the citric acid cycle. As a consequence and with the involvement of the electron transport chain proteins (embedded into the bacteroid membrane), ATP, which provides the energy for nitrogen fixation, is generated. The ammonia that is synthesized is transferred to glutamic acid to form glutamine, which is then transported throughout the plant. Oxygen is slowly released from a complex with bacterial leghemoglobin and is used in several of the above-mentioned enzymatic steps (not shown in this figure). Any excess sugars are converted to polyhydroxybutyrate, a carbon storage polymer, to be used at a later time

commonly used) assay for nitrogenase activity. Component I catalyzes the actual reduction of N_2, and component II donates electrons to component I (Fig. 2.7). Both components are extremely sensitive to oxygen and can be rapidly and irreversibly inactivated when the oxygen concentration is too high. In addition to components I and II, the activity of a complete, functional nitrogenase depends on 15–20

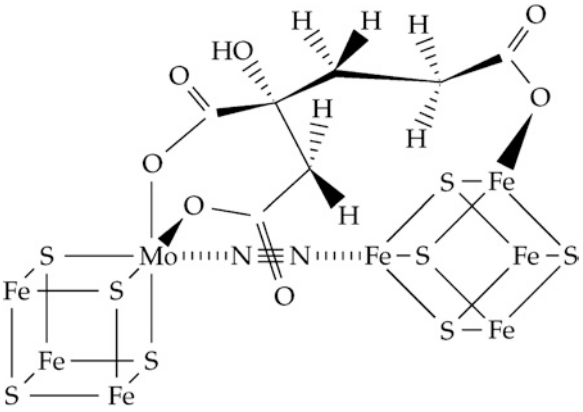

Fig. 2.5 Structure of the iron-molybdenum cofactor (FeMoCo) bound to a molecule of dinitrogen (N_2) prior to its conversion to ammonia. © 2010 American Society for Microbiology. Used with permission. No further reproduction or distribution is permitted without the prior written permission of American Society for Microbiology

Fig. 2.6 Chemical conversion of N_2 (dinitrogen) to NH_3 (ammonia) and acetylene to ethylene

Nitrogen Fixation

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2\uparrow + 16MgADP + 16P_i$$

Acetylene Reduction

$$H{-}C{\equiv}C{-}H + 2H^+ \rightarrow H_2C{=}C{=}CH_2$$

additional accessory proteins. The roles of most of the accessory proteins have been delineated and include the transfer of electrons to component II and the biosynthesis of the iron–molybdenum cofactor that is a part of component I.

Nitrogen fixation is a very complicated process requiring the concerted actions of a large number of different proteins. Therefore, it was not realistic to expect either that an intact single DNA fragment containing all the genetic information for nitrogen fixation could be readily cloned from a diazotrophic microorganism and transferred into a non-diazotrophic organism or that a recipient organism could easily maintain the physiological conditions necessary for nitrogenase activity. In addition, the nitrogenase and many of the other components of this system are highly sensitive to the presence of oxygen so that it is extremely difficult to purify and then biochemically characterize these proteins. Of course, the first time that genes involved in nitrogen fixation (*nif* genes) were isolated, and it was not possible to use previously isolated *nif* genes as DNA hybridization probes. Consequently at that time, the most direct way to isolate *nif* genes was to identify and characterize those clones of a wild-type library that restore nitrogen fixation to various mutants that were unable to fix nitrogen of the original organism. This process is called gene cloning by genetic complementation.

To isolate genes other than those that complement the above-mentioned mutation that are involved in the nitrogen fixation process, isolated *nif* genes have been

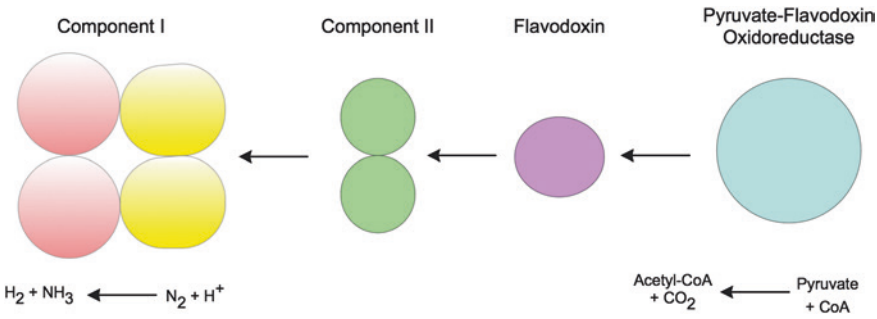

Fig. 2.7 Schematic representation of some of the key reactions in nitrogen fixation. The five polypeptides shown are all part of the nif gene cluster. Nitrogenase consists of component I and component II. Flavodoxin and the pyruvate-flavodoxin oxidoreductase are largely involved in the transfer of electrons to the nitrogenase. The *arrows* between the proteins represent electron flow

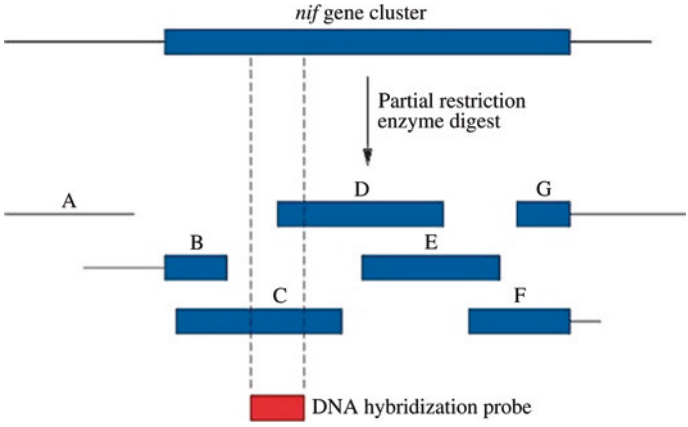

Fig. 2.8 Walking down the chromosome to isolate genes adjacent to an isolated *nif* gene. Partial restriction endonuclease digestion of the region of the chromosomal DNA that encodes *nif* genes. The *nif* gene that was isolated by genetic complementation is used as a DNA hybridization probe is shown in *red*. The DNA hybridization probe and the DNA fragments that result from the partial enzyme digest of the chromosomal DNA are ordered as they would be within the chromosomal DNA. The technique of colony hybridization of a clone bank consisting of the illustrated fragments, using the probe shown, would be expected to identify clones containing plasmids with fragments *C* and *D*. Once these fragments have been isolated and characterized, fragments *C* and *D* may be used, separately, as DNA hybridization probes to screen the same clone bank, in this case identifying clones containing plasmids with fragments *B* and *E*, respectively. In this manner, a large contiguous section of the host chromosome may be isolated as a set of overlapping clones.

used as DNA hybridization probes, which have then been used to screen a chromosomal DNA clone bank that carries large (7–10 kb) inserts (Fig. 2.8). This scheme is based on the observation that in prokaryotic organisms, the genes coding for proteins in a pathway are typically clustered on the chromosomal DNA and

arranged in operons. Thus, DNA hybridization enables investigators to identify clones containing additional *nif* genes that are adjacent to the *nif* gene sequence that was initially isolated by complementation. And, once adjacent genes are identified, they may be characterized by DNA sequencing. In addition to being used to facilitate the isolation of the entire *nif* cluster, this technique of "walking down the chromosome" has been used to isolate nodulation (*nod*) and hydrogenase (*hup*) genes (discussed below).

The entire set of *nif* genes has been isolated and characterized from a number of different bacteria including both *Rhizobia* spp. and numerous rhizospheric and endophytic PGPB. These genes are typically arranged in a single cluster that occupies ~24 kb of the bacterial genome, contains seven separate operons and encodes 20 distinct proteins. All of the *nif* genes are transcribed and translated in a concerted fashion, under the regulatory control of the *nifA* and *nifL* genes, to produce a functional nitrogenase. The NifA protein is a positive regulatory factor. It turns on the transcription of all of the *nif* operons, except its own, by binding to a part of the promoter of each *nif* operon. The NifA protein is an enhancer binding protein that binds to specific DNA sequences located upstream of the *nif* genes and interacts with the transcription initiation protein sigma-54 (σ^{54}) sub-unit of RNA polymerase before transcription from the *nif* promoter is initiated. The NifL protein is a negative regulatory factor; it is a flavoprotein that senses cellular redox status and binds to NifA to block its activity when conditions are not optimal for N_2 fixation. In the presence of either oxygen or high levels of fixed nitrogen, it acts as an antagonist of the NifA protein and, as a result, turns off the transcription of all other *nif* genes. Most diazotrophic organisms have a similar array of genes encoding their nitrogen-fixing apparatus, and the DNA sequences of these genes do not vary much from one organism to another. However, not all nitrogen-fixing organisms have a NifL protein. In some organisms, the essential regions of NifL are part of NifA.

Increasing the amount of nitrogen that an organism can fix also increases the amount of energy, usually in the form of fixed carbon that is needed to power its metabolism. Consequently, a bacterium that has been engineered to fix a higher than normal level of nitrogen may lose its effectiveness as a plant growth-promoting agent because of the diminished growth rate that results from depleting the cell's ATP stores.

Genetic modification of plants with the entire *nif* gene cluster would likely not be effective because the normal level of oxygen in the host cells would inactivate nitrogenase. In addition, it is difficult to conceive how the regulation of nitrogen fixation could be achieved in plants, since there are no plant promoters that respond to the NifA protein. Consequently, *nif* genes would not be turned on in such a transgenic plant. Each of the *nif* genes would also have to be under the control of separate promoters because plant cells cannot process multi-gene transcripts. The introduction of a functional nitrogen fixation capability into plants is therefore extremely unlikely.

While there may not be any straightforward and obvious way to increase nitrogen fixation by manipulating *nif* genes, several indirect strategies are worth considering. For example, since nitrogen fixation is a highly energy intensive process, any genetic manipulation that increases the cell's energy supply might also increase the amount of fixed nitrogen. Normally, in the presence of high levels of carbon (glucose) sources, the excess carbon is converted into glycogen, a carbon storage compound (Fig. 2.9). However, a deletion mutation in the gene that codes for the enzyme glycogen synthase prevents glycogen synthesis so that the glucose enters the tricarboxylic acid (TCA) cycle where the acetyl group of acetyl-CoA is degraded to form carbon dioxide and hydrogen. The hydrogen (or the corresponding electrons) is fed into the electron transport chain and the energy that is released is conserved by phosphorylation of three molecules of ADP to ATP. The

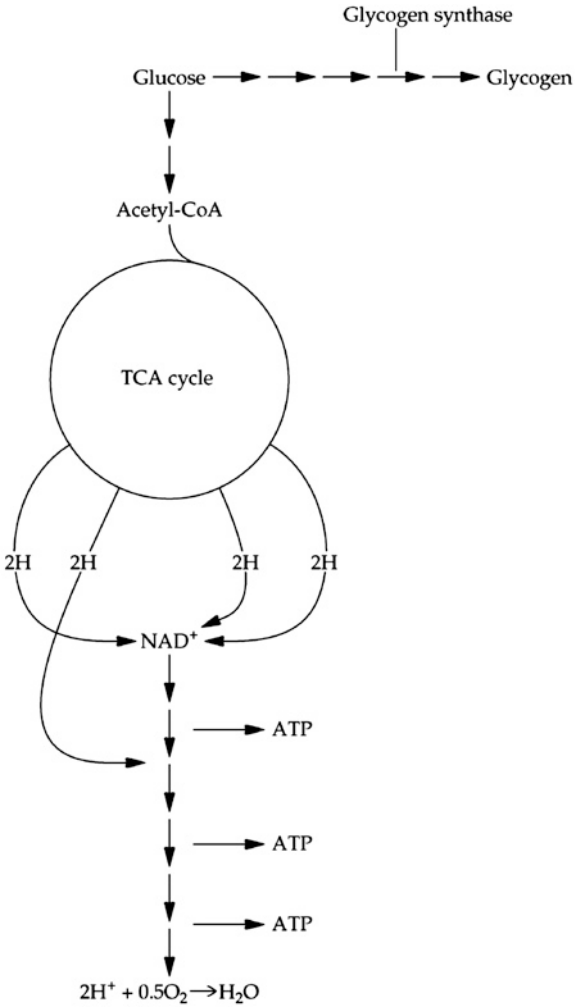

Fig. 2.9 A strategy to indirectly increase nitrogen fixation by increasing the amount of ATP available for nitrogen fixation.

mutated *Rhizobium* increases the nodule number and plant biomass (in the field as well as in the laboratory). Unfortunately, because its metabolism is geared toward "burning off" a significant portion of the carbon intake and synthesizing more ATP, the modified *Rhizobium* strain is less persistent in the soil.

The concentration of oxygen is an important factor in determining the amount of nitrogen that is fixed by a rhizobial strain. On the one hand, oxygen is inhibitory to nitrogenase and is also a negative regulator of *nif* gene expression. On the other hand, oxygen is required for bacteroid respiration. This conundrum can be resolved by the introduction of leghemoglobin, which binds free oxygen tightly so that both the transcription of *nif* genes and the functioning of nitrogenase can proceed unimpaired. In fact, the addition of exogenous leghemoglobin to isolated bacteroids results in a dramatic increase in nitrogenase activity. Thus, it is possible to engineer more efficient strains of *Rhizobium* by overproducing leghemoglobin. Alternatively, since the globin portion of the leghemoglobin molecule is produced by the plant, it may be more efficient to transform rhizobial strains with genes encoding a bacterial equivalent of leghemoglobin.

Following the transformation of a strain of *Rhizobium etli* with a broad-host-range plasmid carrying the *Vitreoscilla* sp. (a gram-negative microaerophilic bacterium) hemoglobin gene at low levels of dissolved oxygen in the growth medium, the rhizobial cells had a 2- to 3-fold higher respiratory rate than the non-transformed strain (Fig. 2.10). In greenhouse experiments, when bean plants were

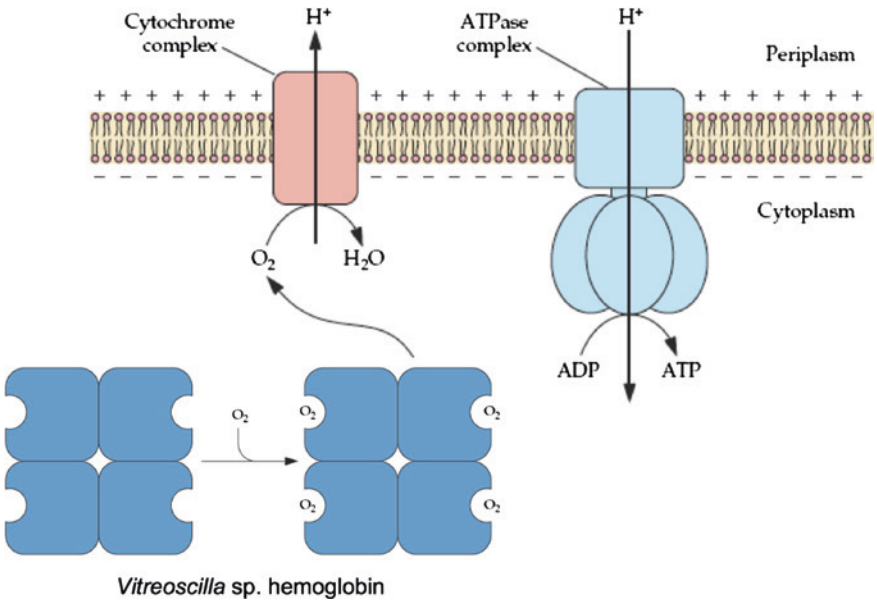

Fig. 2.10 Engineering *Rhizobium etli* to express *Vitreoscilla* sp. hemoglobin gene to bind low levels of dissolved oxygen. Following transformation of *R. etli* with a broad-host-range plasmid carrying the *Vitreoscilla* sp. hemoglobin gene, the *Vitreoscilla* sp. hemoglobin binds oxygen, utilizes the oxygen in pumping H^+ from the cytoplasm to the periplasm, and the H^+ is subsequently taken up thereby generating ATP

inoculated with either non-transformed or bacterial hemoglobin-containing *R. etli,* the plants inoculated with the hemoglobin-containing strain had approximately 68 % more nitrogenase activity. This difference in nitrogenase activity led to a 25–30 % increase in leaf nitrogen content and a 16 % increase in the nitrogen content of the seeds that are produced. It remains to be seen whether this genetically modified strain is as effective in the field as in the greenhouse and whether the regulatory authorities will approve its use in the field.

In Latin America, a majority of the bean plants are nodulated by *R. etli*. One strain of *R. etli* that is in common use in Latin America encodes three copies of the nitrogenase component II, which is also called nitrogenase reductase, encoded by the *nifH* gene(s), with each of these genes under the transcriptional control of a separate promoter. To increase the amount of nitrogenase in *R. etli*, the strongest of the three *nifH* promoters (i.e., *PnifHc*) was coupled to the *nifHcDK* operon, which encodes the nitrogenase structural genes (where *nifHc* is one of the three *nifH* genes in this bacterium). The *nifHc* promoter is typically induced during nodule development. The *PnifHc–nifHcDK* construct was cloned into a broad-host-range plasmid and introduced into the wild-type strain of *R. etli*. The net result of this genetic manipulation was a significant increase in nitrogenase activity, plant dry weight, seed yield, and the nitrogen content of the seeds (Table 2.2). Moreover, this genetic manipulation worked as well or better when the *PnifHc–nifHcDK* construct was introduced into the large Sym plasmid from *R. etli* that contains all of the genetic information for nodulation and nitrogen fixation.

Biological nitrogen fixation requires a large amount of energy in the form of ATP (Fig. 2.6). Thus, any mutation or genetic manipulation that increases the flux of carbon sources consumed by a bacterium through the citric acid cycle should be beneficial for nitrogen fixation (Fig. 2.11). This is because metabolism of glucose via the citric acid cycle results in the production of ATP. Consistent with this principle, it was observed that expression of the *PnifH$_c$–nifH$_c$DK* construct in a poly-β-hydroxybutyrate-negative strain of *R. etli* enhanced plant growth to an even greater extent than when this construct was expressed in a wild-type poly-β-hydroxybutyrate-positive strain. Finally, since no foreign genes were introduced

Table 2.2 Symbiotic behavior of genetically modified strains of *R. etli* in concert with common beans (*Phaseolus vulgaris*)

Bacterial strain	Nitrogenase activity, mmol ethylene/h/g of nodule	Plant dry weight, g/plant	Seed yield, g/plant	Seed N content, mg of N/g of seed
Wild-type	64.5	0.54	1.43	33.9
Wild-type + extra *nifHDK*	72.7	0.66	1.56	41.4
Wild-type + *nifH$_c$*	77.3	0.75	1.73	31.2
Wild-type + *PnifH$_c$–nifH$_c$DK*	108.2	0.81	2.50	43.6

Adapted from Peralta et al., *Appl. Environ. Microbiol.* **70**:3272–3281, 2004

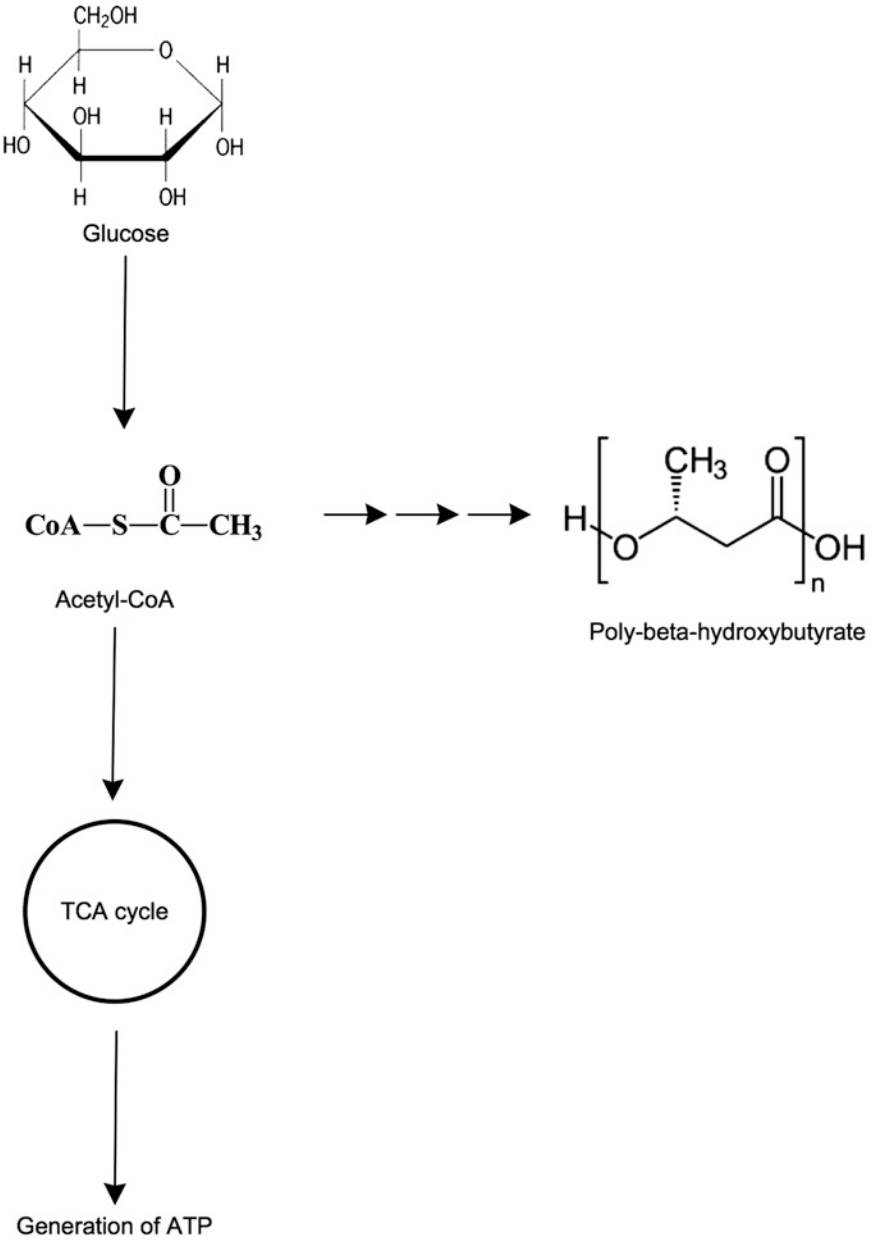

Fig. 2.11 Conversion of glucose to acetyl-CoA that in turn may either be converted into poly-β-hydroxybutyrate or used to generate ATP

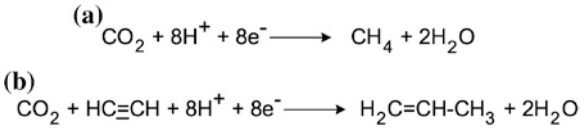

Fig. 2.12 **a** Conversion of carbon dioxide to methane by a modified nitrogenase. **b** Conversion of carbon dioxide and acetylene to propylene by a modified nitrogenase

into *R. etli,* the scientists who constructed these strains hope that the regulatory bodies in their country will view the manipulated strains as benign and approve them for widespread environmental use.

As a direct result of worldwide industrialization and the burning of fossil fuels, the amount of carbon dioxide in the atmosphere has been increasing for the past 100 years or so and contributing to global warming. Recently, researchers reported that by directed mutagenesis they were able to alter the specificity of the nitrogenase enzyme so that it was able to reduce carbon dioxide to methane (Fig. 2.12a). This change in the specificity of nitrogenase occurred as a consequence of changing two amino acid residues in the alpha sub-unit of component I; the Val at position 70 was changed to Ala and the His at position 195 was changed to Gln. These two amino acid substitutions altered the protein environment around the FeMo cofactor. These amino acid changes were made on the basis of previous experiments that showed that the protein environment immediately surrounding the FeMo cofactor control both the size of compounds that can be nitrogenase substrates and the reactivity of the FeMo cofactor toward those compounds. In addition to converting carbon dioxide to methane, the modified nitrogenase was able to catalyze the synthesis of propylene through the reductive coupling of carbon dioxide and acetylene (Fig. 2.12b). These chemical reactions occur at faster rates than any other enzymatic conversions of carbon dioxide to methane and require only a single enzyme, whereas other systems utilize a consortium of enzymes. However, the modified nitrogenase, like the native enzyme, does use considerable energy in the form of ATP. This work is only the first step in what is hoped will eventually be an efficient and clean means of converting unwanted greenhouse gases (carbon dioxide) into industrially useful compounds.

2.1.2.2 Nodulation

A major goal of agricultural biotechnology research is the development, by genetic manipulation, of *Rhizobium* strains that can increase plant productivity more effectively than naturally occurring strains. In this regard, many commercial inoculant strains that have been developed by traditional mutation and selection to be superior nitrogen fixers are not very effective at establishing nodules on host plant roots when placed in competition with *Rhizobium* strains that are already present in the soil. Conversely, although many of the strains that are indigenous to the soil are highly successful in establishing nodules in competitive situations,

they are often not especially efficient at nitrogen fixation. It would therefore be advantageous to be able to increase the nodulation capability of commercial inoculant strains that were previously selected for their ability at nitrogen fixation. The question then arises, what is the genetic basis of this effective "competitiveness" at nodulation.

When scientists first attempted to isolate nodulation (*nod*) genes, the absence of any specific information about the biochemical or genetic basis of nodulation meant that a strategy had to be devised for the identification of the genes. Therefore, the technique of genetic complementation was used. Nodulation-defective (Nod^-) mutants of *Sinorhizobium meliloti* (a strain that nodulates alfalfa, *Medicago*; sweet clover, *Melilotus*; and fenugreek, *Trigonella*) were transformed with a clone bank of wild-type *S. meliloti* chromosomal DNA, and those colonies that had acquired the ability to nodulate alfalfa roots were isolated. Once a single nodulation gene was identified, it was used as a DNA hybridization probe to "walk down the chromosome" and identify adjacent regions of the *S. meliloti* chromosomal DNA.

The isolation and characterization of the complete repertoire of nodulation genes from *S. meliloti* (and subsequently other rhizobial species) revealed that, similar to nitrogen fixation, nodulation and its regulation is a complex process that requires the functioning of a large number of genes. Some nodulation genes are highly conserved (i.e., common) among nodulating bacteria, and others are species specific. The *nod* genes are grouped into three separate classes: common genes, host-specific genes, and the regulatory *nodD* gene. Thus, for example, the *nodABC* genes are common to all *Rhizobium* species, are structurally interchangeable, and in most species they are found on a single operon.

The bacterium *S. meliloti* has been shown to utilize chemotaxis to optimize its movement toward host plant-secreted nutrients that act as chemo-attractants. This enables this bacterium (and others) to locate infection sites along emerging roots and, as a result, to compete more effectively for nodulation. Germinating seeds exude a wide range of organic compounds, and alfalfa seed exudates elicited a chemotactic response from *S. meliloti* (Fig. 2.13). The early recruitment of the microbial symbiont to the growing root, e.g., during seed germination, is one strategy for maximizing the rhizobia–legume interaction. Earlier research had suggested that flavonoids act as host-specific chemo-attractants; however, they elicit only a weak chemotactic response and have only a short diffusion range in aqueous soil due to their hydrophobic nature. Thus, amino acids, organic acids, and sugars are more likely to function as recruiting agents, thereby facilitating the initial binding of the bacterium to the plant.

Subsequently, a number of events occur during nodulation (Fig. 2.14a). First, the *nodD* gene product, which is constitutively expressed, recognizes and binds to a flavonoid molecule, which is excreted by the roots of the potential host plants. Flavonoids are a class of plant secondary metabolites that perform a number of different functions for the plant, such as pigmentation and defense against fungi or insects. The binding of flavonoids to the NodD protein is the major determinant of rhizobial host specificity, because each rhizobial species recognizes only a limited

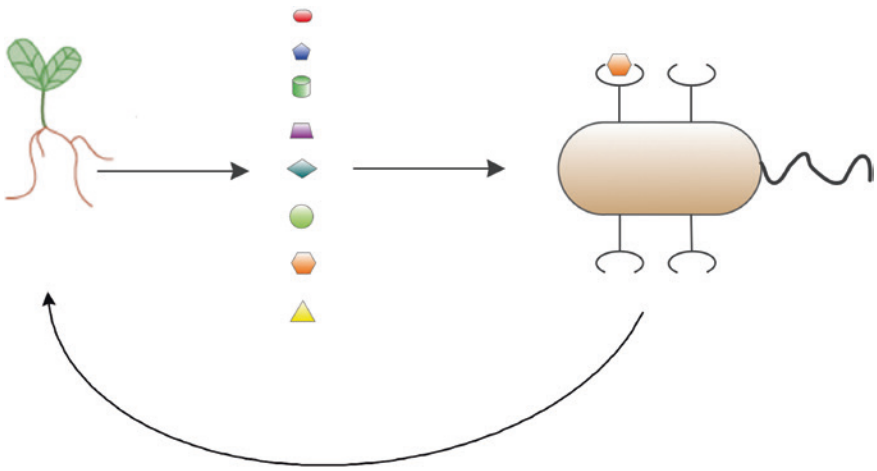

Fig. 2.13 Binding of small molecule chemo-attractants secreted by plants by bacterial receptors—eight different chemo-attracts exuded by alfalfa plants that can bind to *Sinorhizobium meliloti* receptors have been identified—and the subsequent movement of the bacteria toward the plant and the sites of chemo-attractant secretion

number of flavonoid structures and each plant species produces its own specific set of flavonoid molecules (Table 2.3). Some strains, such as *R. leguminosarum* biovar (bv.) trifolii, have a very narrow host range, responding to only a few kinds of flavonoids, while others, such as *Rhizobium* sp. strain NGR234, have a very broad host range and respond to a much larger number of different flavonoids.

The binding of a flavonoid molecule activates the NodD protein and enables the flavonoid–NodD complex to attach to a nodulation promoter element called a *nod* box (Fig. 2.14b). This promoter element is located upstream from all the nodulation genes except the *nodD* gene, and it activates the transcription of these genes. The *nodABC* genes encode proteins that cause the plant root hair tips to swell and curl, the initial step in the infection of the plant root by the bacterium. The bacteria synthesize an oligosaccharide nod factor (Fig. 2.15) that elicits in the plant a host-specific response that includes root hair curling and deformation and is essential for *Rhizobium* to induce nodules. After the initial change in the root morphology, the bacterium attaches to the root hair. Next, the bacterial cell penetrates the plant cell through an infection thread. As the bacterial cell divides, it moves through the infection thread. Finally, a number of additional *nod* gene products are synthesized. These proteins, together with some plant-encoded proteins, contribute to the formation of the nodule.

It has become clear that the process of nodulation is quite complicated. Thus, considerable additional effort will be required before it is possible to further enhance the competitiveness of rhizobial strains by genetic engineering. To date, despite the fact that *nod* genes have been isolated and characterized from numerous rhizobial strains, no simple genetic means has been devised for using *nod*

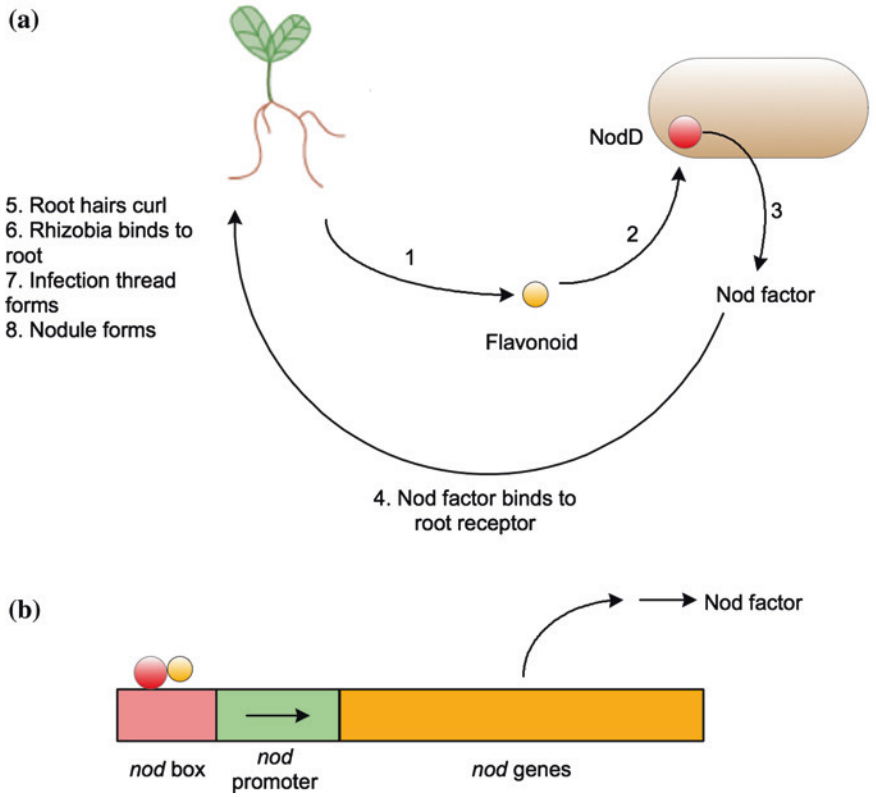

Fig. 2.14 Overview of the nodulation process. **a** The plant secretes/releases specific flavonoid molecules (*1*), the flavonoid is taken up and bound to the NodD protein (*2*), the flavonoid-NodD complex activates the synthesis of the Nod factor (*3*), Nod factor binds to a root receptor (*4*), the root hair tips swell and curl (*5*), the *Rhizobium* attaches to the root hair tips (*6*), an infection thread forms (*7*) and once inside the root cells, the nodule forms (*8*). **b** The flavonoid-NodD complex binds to the *nod* box that is a part of the *nod* promoter and activates the synthesis of the Nod factor and transcription of other *nod* genes

genes to enable inoculated strains of *Rhizobium* to outcompete indigenous strains. Nevertheless, it is possible to alter host specificity by the transfer of a *nodD* gene from a broad-specificity rhizobial strain to one with narrow specificity.

Ethylene is often produced by a plant following the plant's infection by a soil microorganism such as a strain of rhizobia. Thus, nodule formation engenders a small rise in the plant ethylene level that is generally localized to a portion of the root and can inhibit, and therefore limit, subsequent rhizobial infection and nodulation. One way in which some strains of *Rhizobium* naturally increase the number of nodules that they can form on the roots of a host legume is to limit the rise in ethylene that occurs following the initial infection. Different *Rhizobium* species decrease ethylene levels either by synthesizing a small molecule called rhizobitoxin that chemically inhibits ACC synthase, one of the ethylene biosynthetic

Table 2.3 Some legumes and the *nodD* gene inducers that they produce

Legume	Compound
Lupin (*Lupinus albus*)	Erythronic acid, tetronic acid
Alfalfa (*Medicago sativa*)	Stachydrine, trigonelline, luteolin, chrysoeriol, 4,4′-dihydroxy-2′-methoxychalcone, liquiritigenin, 7,4′-dihydroxyflavone
Clover (*Trifolium repens*)	7,4′-Dihydroxyflavone, geraldone, 4′-hydroxy-7-methoxyflavone
Common bean (*Phaseolus vulgaris*)	Delphinidin, kaempferol, malvidin, myricetin, petunidin, quercetin, eriodictyol, genistein, naringenin
Pea (*Pisum sativa*)	Apigenin, eriodictyol
Soybean (*Glycine max*)	Daidzein, genistein, coumesterol
Vetch (*Vicia sativa* subsp. *nigra*)	3,5,7,3′-Tetrahydroxy-4′-methoxyflavanone, 7,3′-dihydroxy-4′-methoxyflavanone, naringenin, 4,4′-dihydroxy-2′-methoxychalcone, liquiritigenin, 7,4′-dihydroxy-3′-methoxyflavanone, 5,7,4′-trihydroxy-3′-methoxyflavanone, 5,7,3′-trihydroxy-4′-methoxyflavanone naringenin

These inducers are commonly released by either roots or germinating seeds

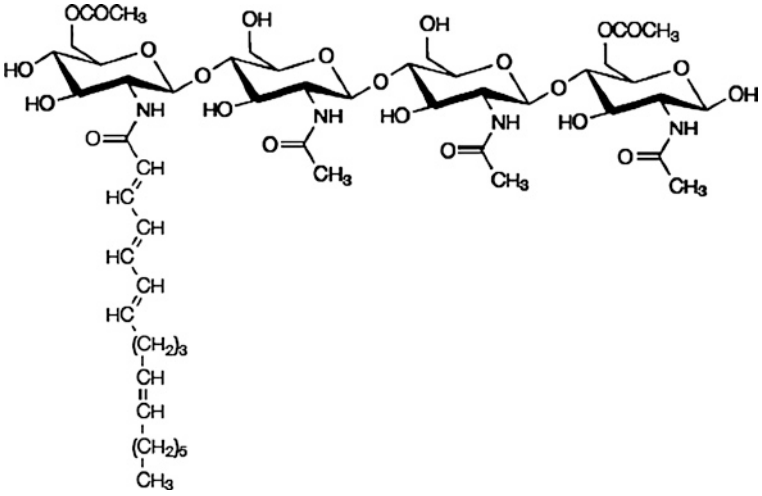

Fig. 2.15 Chemical structure of a Nod factor from *Rhizobium leguminosarum* bv. viciae. © 2010 American Society for Microbiology. Used with permission. No further reproduction or distribution is permitted without the prior written permission of American Society for Microbiology

enzymes, or by producing the enzyme ACC deaminase and cleaving and hence removing some of the ACC before it can be converted to ethylene. The result of lowering the local level of ethylene in plant roots is that both the number of nodules and the biomass of the plant are increased by 25–40 %. Assays of isolated rhizobia indicate that in the field approximately 1–10 % of rhizobial strains naturally possess ACC deaminase. It should therefore be possible to increase the nodulation efficiency of *Rhizobium* strains that lack ACC deaminase by genetically engineering these strains with isolated *Rhizobium* ACC deaminase genes

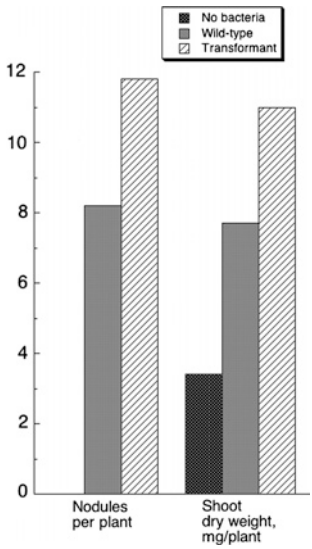

Fig. 2.16 Increased ability of *Sinorhizobium meliloti* transformed with an ACC deaminase gene (and its regulatory region) from *Rhizobium leguminosarum* bv. viciae to nodulate alfalfa (nodules per plant) and promote plant growth (shoot dry weight in mg per plant)

(and their regulatory regions). In fact, insertion of a single copy of an ACC deaminase gene from *R. leguminosarum* bv. *viciae* into the chromosomal DNA of a strain of *S. meliloti* that lacked this enzyme dramatically increased both the nodule numbers and biomass of host alfalfa plants (Fig. 2.16). Although genetically engineered strains of *Rhizobium* are generally not acceptable for use in the field in most jurisdictions throughout the world at this time, as a result of this work, several commercial inoculant producers have recently begun screening newly isolated *Rhizobium* strains for active ACC deaminase.

In addition to insuring that rhizobial inoculants contain ACC deaminase and thereby insuring an increased number of nodules and a higher plant biomass, dual inoculation of pea plants with strains of *R. leguminosarum* and *Pseudomonas putida* synergistically improves plant growth, nodulation, and seed yield (Table 2.4). In this case, despite the fact that both strains contained ACC deaminase, the presence of the two strains always produced the largest and healthiest plants and the greatest yield of peas. Moreover, this inoculation was more effective

Table 2.4 Growth of pea plants in the field following different treatments

Treatment	Dry weight		N content		Seed yield, g/plant
	Roots, g/plant	Shoots, g/plant	Roots, mg/g	Shoots, mg/g	
Control	0.35	1.2	30.0	40.0	5.4
P. putida	0.55	1.8	32.0	46.3	7.8
R. leguminosarum	0.65	1.8	32.3	48.0	8.2
P. putida + *R. leguminosarum*	0.80	2.3	39.5	59.3	10.2
N + P fertilizer	0.85	2.1	38.3	52.7	9.2

Adapted from Ahmad et al. 2013. Symbiosis 61:93–104

than the addition of optimal levels of nitrogen and phosphorus fertilizer, and this effect was significant both in the greenhouse and in the field. Thus, not only can rhizobial inoculants be improved by selecting for the presence of ACC deaminase, but also additional growth improvements are possible when a dual inoculation strategy is employed.

2.1.2.3 Hydrogenase

An undesirable side reaction of the fixation of nitrogen by nitrogenase is the reduction of H^+ to hydrogen gas (H_2) (Fig. 2.17). In this reaction, energy in the form of ATP is wasted on the production of hydrogen, which is eventually lost to the atmosphere. This side reaction significantly lowers the overall efficiency of the nitrogen-fixing process. However, if H_2 could be recycled to H^+, the extent of energy loss could be diminished and the nitrogen-fixing process would become more efficient. This side reaction cannot be prevented directly because it is a consequence of the chemistry of the active site of the nitrogenase; hence, it is not possible to alter nitrogenase as a means of blocking hydrogen production.

On the other hand, some strains of *Bradyrhizobium japonicum* (as well as other *Rhizobia*) have an enzyme called hydrogenase that can take up H_2 from the atmosphere and convert it into H^+ and in the process produce ATP that can be used to fix more nitrogen (Fig. 2.17). In addition, plants inoculated with strains that produce hydrogenase (they are said to be hydrogen uptake positive or Hup^+) have more biomass and nitrogen than plants that are treated with strains that do not produce hydrogenase (i.e., they are Hup^-), independent of the level of nitrogenase activity in the Hup^- strains (Table 2.5). Thus, the presence of a hydrogen uptake system in a symbiotic diazotroph, such as *B. japonicum,* improves the ability of a bacterium to stimulate plant growth, presumably by binding and then recycling

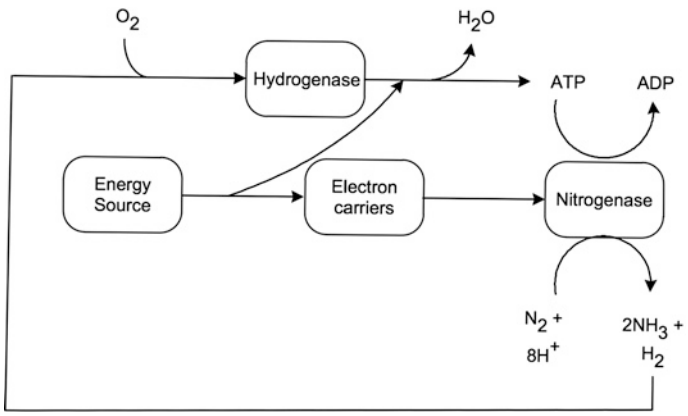

Fig. 2.17 Recycling of the hydrogen gas that is produced as a by-product of nitrogen fixation. When it is present, hydrogenase captures the hydrogen gas and converts it into H^+ and ATP

Table 2.5 Relative enzyme activities and growth-stimulating performance of a parental Hup^+ *B. japonicum* strain (SR) and three Hup^- mutants of the same strain (SR1, SR2, and SR3)

B. japonicum strain	Relative nitrogenase activity	Relative hydrogenase activity	Relative plant dry weight	Relative nitrogen content
SR	1.00	1.00	1.00	1.00
SR1	1.27	0.01	0.81	0.93
SR2	1.13	0.01	0.74	0.91
SR3	1.23	0.01	0.65	0.85

Adapted from Albrecht et al., *Science* **203:**1255–1257, 1979. Nitrogenase activity was assessed by monitoring the amount of acetylene that was reduced to ethylene by gas chromatography as a function of time. Hydrogenase activity was measured by means of a hydrogen electrode. Plant dry weight included the entire plant. The nitrogen content was calculated as the fraction of the plant dry weight that was nitrogen. All values have been normalized relative to the parental strain

the H_2 that is formed inside the nodule by the action of nitrogenase (Fig. 2.17). Although it is clearly beneficial to the plant to obtain its nitrogen from a symbiotic diazotroph that has a hydrogen uptake system, this trait is not commonly found in naturally occurring rhizobial strains so that Hup^- rhizobial strains are prime candidates for genetic transformation to a Hup^+ phenotype.

However, the conversion of a Hup^- strain of *Rhizobium* into a Hup^+ strain may not be readily achieved by the introduction of just a single hydrogenase gene. Rather, the introduced gene(s) must encode all of the enzyme's sub-units and must be able to interact with the appropriate electron transport molecule within the host organism.

The most common strategy for isolating hydrogenase genes has been genetic complementation followed by walking down the chromosome. In this regard, *hup* genes from *B. japonicum* were isolated from a clone bank of wild-type DNA constructed in the broad-host-range cosmid vector pLAFR1 by complementation of *B. japonicum* Hup^- mutants. The presence of a hydrogenase that takes up hydrogen from the atmosphere in the complemented Hup^- mutant strains was indicated by the ability of the active hydrogenase to reduce the dye methylene blue in a hydrogen atmosphere and thereby change its color. Detailed studies of the *B. japonicum hup* genes showed that they were organized into several transcriptional units covering approximately 20 kb of the genome and including 18 separate genes. Subsequent studies, on the *hup* genes from *Rhizobium leguminosarum,* have indicated that these genes are similar in both DNA sequence and gene organization to the *hup* genes from *B. japonicum*.

Following the isolation of the complete complement of *R. leguminosarum hup* genes, and despite the complexity of this system, it is possible to use cosmid vectors to transfer all of these genes, originally from a naturally occurring Hup^+ strain of *R. leguminosarum*, to a naturally occurring Hup^- strain (Table 2.6). Plants treated with *R. leguminosarum* that had been transformed to Hup^+ grew larger and contained more nitrogen than the plants inoculated with the Hup^- parental strain. Thus, the ability of a diazotroph to stimulate plant growth may be improved, albeit directly, by genetic manipulation.

Table 2.6 Plant growth and nitrogen assimilation after the introduction of the complete panel of *R. leguminosarum hup* genes into a Hup$^-$ strain of *R. leguminosarum*

Bacterial phenotype	Relative plant dry weight	Relative nitrogen amount	Relative leaf area	Relative nitrogen concentration
Hup$^-$	1.00	1.00	1.00	1.00
Hup$^+$	1.35	1.52	1.53	1.15

Adapted from Brewin and Johnston, US patent 4,567,146, January 1986. The data have been normalized relative to the Hup$^-$ parental strain

In *R. leguminosarum,* 17 genes are associated with hydrogenase activity. There are ten *hup* genes (*hup SLCDEFGHIJK*) responsible for the structural components of the hydrogenase, the processing of the enzyme, and electron transport (Fig. 2.18). There are also seven *hyp* (hydrogenase pleiotropic) genes (*hypABFCDEX*), which are involved in processing the nickel that is part of the active center of the enzyme. The *hup* promoter is dependent on the NifA protein (which is also required to activate the synthesis of *nif* genes), so that *hup* genes are expressed only within bacteroids. On the other hand, the *hyp* genes are transcriptionally regulated by an FnrN-dependent promoter (Fnr = fumarate-nitrate reduction regulator), which is switched on when the cellular levels of oxygen are low (i.e., under microaerobic conditions). Thus, the *hyp* genes are typically expressed in bacteroids.

Modifying the chromosomal DNA of *R. leguminosarum* and exchanging the *hup* promoter for an FnrN-dependent promoter (Fig. 2.18) enabled researchers to

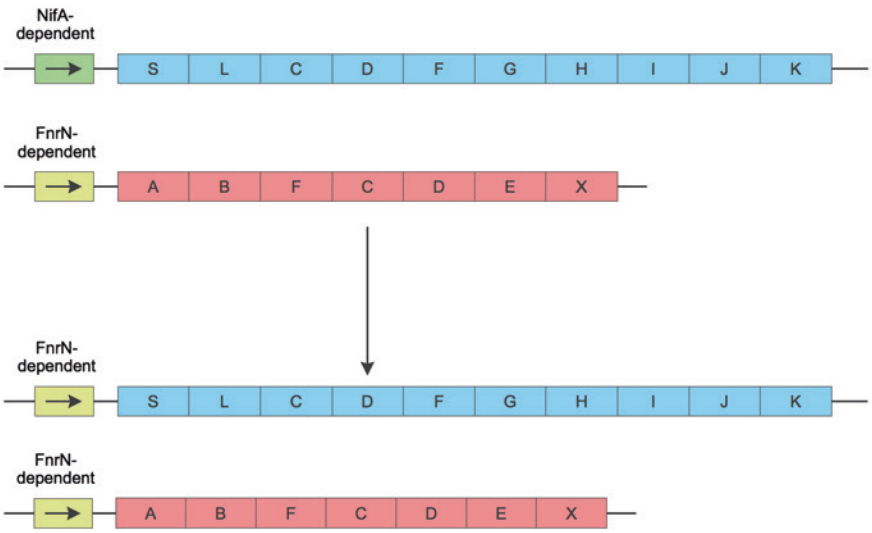

Fig. 2.18 Replacement of the *R. leguminosarum* NifA-dependent *hup* promoter with the *R. leguminosarum* FnrN-dependent *hyp* promoter so that the *hup*(*SLCDEFGHIJK*) genes are controlled by the *hyp* promoter. The *R. leguminosarum hyp* genes (i.e., *hyp*(*ABFCDEX*)) are already under the transcriptional control of the FnrN-dependent *hyp* promoter. The *arrows* indicate the direction of transcription

Table 2.7 Hydrogenase activity in bacteroids and hydrogen evolution from nodules with different legume host plants in symbiosis with *Rhizobium leguminosarum* bv. *viciae*

Legume	Common name	Relative hydrogenase activity	Relative hydrogen evolution
Pisum sativum	Pea	140	0.095
Lens culinaris	Lentil	1	1
Lathyrus sativus	White pea	80	0.02
Lathyrus odoratus	Sweet pea	45	0.615
Vicia sativa	Common vetch	87	0.095
Vicia villosa	Hairy vetch	382	0.045
Vicia ervilia	Bitter vetch	92	0.002
Vicia monanthos	Single-flowered vetch	473	0.024

Both hydrogenase activity and hydrogen evolution values have been normalized to the value reported for *Lens culinaris*. Adapted from Brito et al. (2008) Molec Plant Microbe Interact 21:597–604

create a derivative of the wild-type bacterium with an increased level of hydrogenase activity. The engineered *R. leguminosarum* strain displays a twofold increase in hydrogenase activity compared to the wild-type and a sixfold decrease in the amount of H_2 that is produced as a by-product of nitrogen fixation. In the transformed strain, the amount of hydrogen evolved from nitrogen-fixing nodules was extremely low, indicating that virtually all of the hydrogen produced by nitrogenase was recycled. This is expected to make this strain of *R. leguminosarum* much more effective at promoting plant growth and increasing plant nitrogen content. When the effectiveness of the engineered strain of *R. leguminosarum* bv. *viciae* was tested with a variety of different legume hosts, all of the legumes tested with the notable exception of lentils displayed an increased level of hydrogen uptake hydrogenase activity in bacteroids and as a consequence a decreased level of hydrogen evolution (Table 2.7). At this point, it is not clear why different legume hosts respond so differently to the presence of the introduced *R. leguminosarum.*

2.2 Sequestering Iron

Living organisms, animals, plants, and microorganisms, all require iron as a component of proteins involved in important processes such as respiration, photosynthesis, and nitrogen fixation. Despite the abundance of iron on the earth's surface, soil organisms such as plants and microbes cannot readily assimilate enough iron to support their growth because the iron in soil is largely present as insoluble, ferric (Fe^{+3}) hydroxides, which is only sparingly soluble and cannot be readily transported into cells. To solve this problem, bacteria, fungi, and some plants secrete low molecular mass (~400–1,000 Da), specialized iron-binding molecules called siderophores into the soil to scavenge iron. Siderophores bind to Fe^{+3} with an

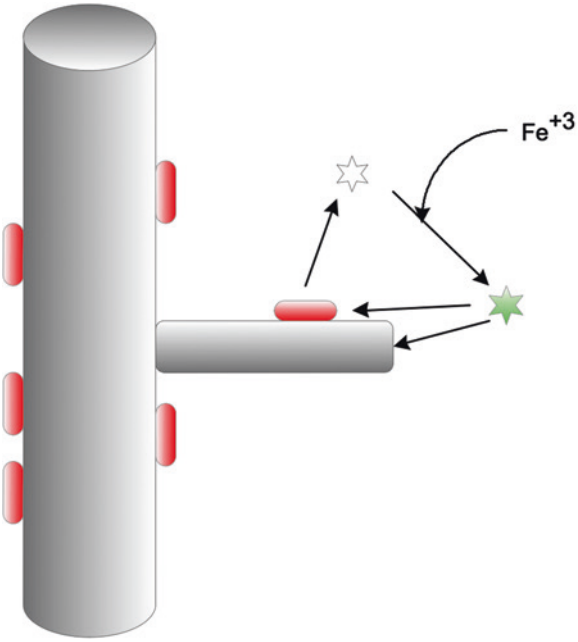

Fig. 2.19 Schematic representation of bacterial siderophores (*star shape*) sequestering iron from the environment. The siderophore–iron complex may be taken up either by the siderophore-synthesizing bacteria or by plants

exceptionally high affinity (i.e., Kd = 10^{-20} to 10^{-50}). Once bound, the now soluble iron–siderophore complex is taken up by specific receptors on the exposed surfaces of microorganisms or plants (Fig. 2.19); following reduction to the ferrous state (Fe^{+2}), the iron is released from the siderophore.

The promotion of plant growth by siderophore-producing bacteria occurs by either directly supplying iron for plant utilization and/or by removing iron from the environment of phytopathogens, thereby reducing their competitiveness.

2.2.1 Siderophore Structure

Siderophores are low molecular weight molecules, usually less than 1 kDa, with three functional, or iron-binding, groups connected via a flexible backbone (Fig. 2.20). Each functional group has two oxygen atoms, or less commonly, nitrogen, that bind to iron. The functional groups are bidentate, and trivalent ferric iron can accommodate three of these groups to form a six-coordinate complex. The functional groups on microbial siderophores are typically hydroxamates or catecholates; however, other functional groups including carboxylate moieties such as citrate and ethylenediamine are also commonly used (Fig. 2.21). Different combinations of these functional groups may be present on a single siderophore molecule. In general, hydroxamate-type siderophores are typical to fungi, while catecholates, which bind iron more tightly than hydroxamates, are common in

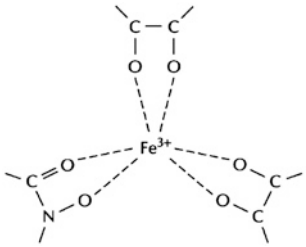

Fig. 2.20 A six coordinate iron–siderophore complex. Three bidentate functional groups on a siderophore molecule bind to the ferric iron.

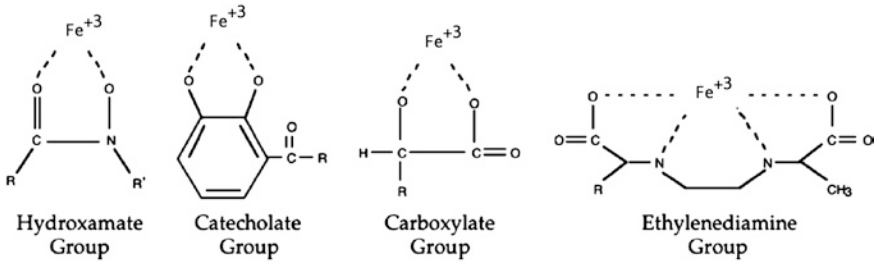

Fig. 2.21 Iron-binding groups that may be present in a microbial siderophore.

bacterial siderophores. Plant siderophores, which bind iron much less tightly than microbial siderophores, are linear hydroxy- and amino-substituted iminocarboxylic acids, such as mugineic acid and avenic acid. Although many molecules with negatively charged donor groups are able to bind to iron, they do so with a much lower affinity than bacterial siderophores. Other trivalent metal ions, such as aluminum, also bind bacterial siderophores, albeit with a lower affinity. Most environmental metals, however, are divalent and less electronegative and therefore do not bind tightly to bacterial siderophores.

Enormous structural diversity exists among the hundreds of known siderophores, even among the multiple siderophores synthesized by a single organism. Thus, for example, various *Psuedomonas* spp. produce two major types of siderophores, pyochelin and pyoverdin (Fig. 2.22). Pyochelins are phenolate siderophores derived from salicylic acid and cysteine; two pyochelin molecules bind one molecule of ferric iron with a relatively low efficiency. Pyoverdins have a much greater affinity for iron; they are water-soluble pigments that fluoresce yellow-green under ultraviolet light giving the fluorescent pseudomonads their characteristic appearance.

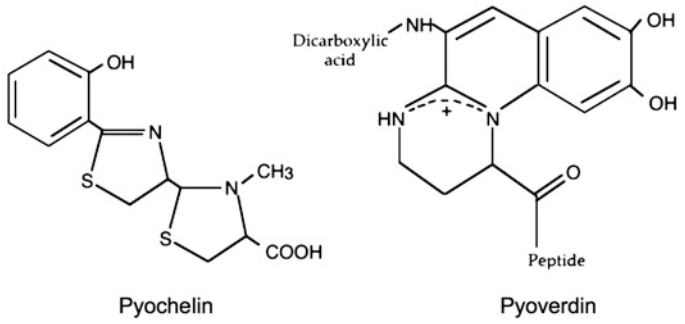

Fig. 2.22 Structures of two well-characterized classes of *Pseudomonas* spp. siderophores

2.2.2 Siderophore Biosynthesis Genes

Given the structural diversity that exists between siderophores from different bacteria, the isolation of siderophore biosynthesis genes is not necessarily simple or straightforward. One reliable approach to the isolation of siderophore biosynthesis genes utilizes the strategy of cloning by complementation (discussed earlier). Thus, it is necessary to first isolate a series of mutants in siderophore biosynthesis. In one instance, the PGPB *P. putida* WCS358 was first mutagenized and 28 siderophore-deficient mutants were selected by (i) lack of fluorescence under ultraviolet light and (ii) the inability to grow in the presence of bipyridyl, a molecule that sequesters most of the iron in the growth medium. When most of the iron is unavailable, only a cell that produces siderophores can grow.

A clone bank of *P. putida* WCS358 DNA was then constructed in the broad-host-range cosmid vector pLAFR1 and was introduced by conjugation into each of the 28 siderophore-deficient mutants (Fig. 2.23). All of the resultant transformants were tested by complementation for restoration of fluorescence and/or the ability to grow in the presence of bipyridyl. Thirteen separate complementing cosmid clones, with an average insert size of 26 kb, were identified. Following detailed analyses, these clones were found to represent at least five separate gene clusters. When one of these gene clusters was studied in detail, it was found to have a length of 33.5 kb and to contain five transcriptional units with at least seven separate genes. Additional siderophore biosynthesis genes were isolated using the technique of walking down the chromosome described earlier. Thus, similar to nitrogen fixation and nodulation, siderophore biosynthesis is a complex process involving a number of different genes. Therefore, genetically engineering bacteria to produce modified siderophores is not a simple matter. However, it may be possible to extend the range of iron–siderophore complexes that one bacterial strain can utilize so that a genetically altered plant growth-promoting biocontrol bacterial strain could take up and use siderophores synthesized by other soil

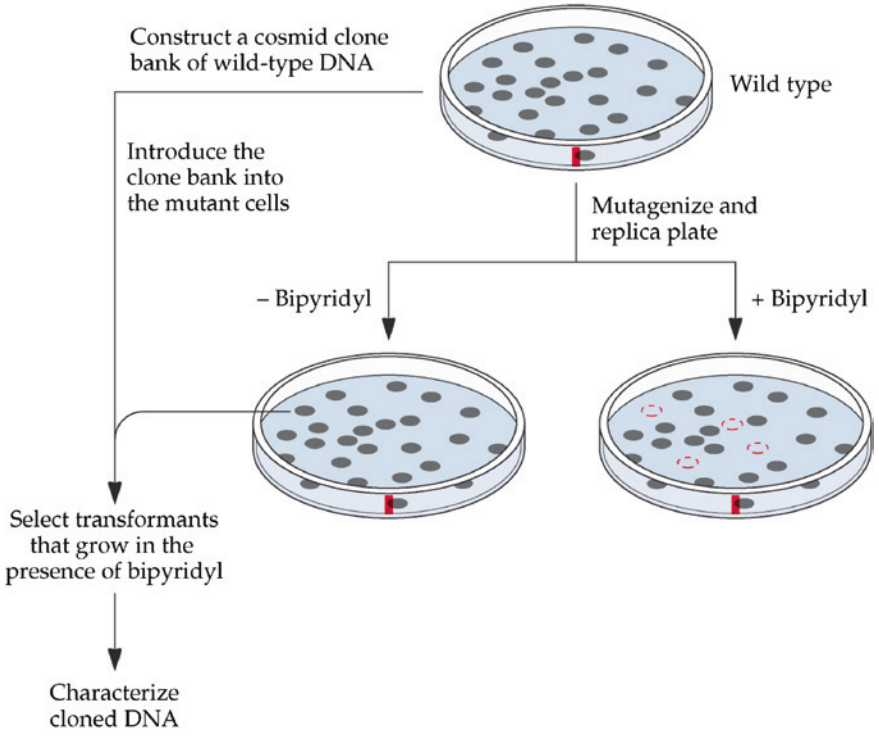

Fig. 2.23 Isolation of genes involved in siderophore biosynthesis. Mutants unable to grow on bipyridyl (because they do not produce siderophores and therefore cannot take up the trace amounts of iron in the medium in competition with the bipyridyl) were complemented using a clone bank of wild-type DNA in the broad-host-range cosmid pLAFR1. Transformants that can grow in the presence of bipyridyl do so because they can complement the mutation in one of the siderophore biosynthesis genes.

microorganisms, thereby giving it a competitive advantage. This was achieved when the genes for iron–siderophore receptors from one PGPB were isolated and then introduced into other PGPB strains.

2.2.3 Regulation of Iron Uptake

The bound iron must also be taken up and released into bacterial or plant cells to become available for their metabolism. Because siderophores are hydrophilic, they rely on membrane-bound proteins for transportation across cell membranes. Once inside the cell, iron is released from the siderophore–iron complex to be available for use in metabolic processes. For this to happen, either the siderophore is

enzymatically cleaved or the ferric iron is reduced to the ferrous state. To this end, researchers have identified (i) some esterases that can cleave siderophores (and release iron as a consequence of reduced affinity) and (ii) some reductases that can reduce bound ferric iron (which is then released from the siderophore–iron complex).

Transcription of most of the genes whose products are involved in siderophore synthesis and iron transport is activated under conditions of iron limitation. In pseudomonads, the cellular concentration of iron is sensed by "Fur," an iron-binding protein. When cellular levels of iron are sufficient, this cytoplasmic protein binds ferrous iron and represses the transcription of iron-regulated genes. A Fur-binding consensus sequence, called the Fur-box, has been found in the promoter regions of a number of *Pseudomonas* (and other PGPB) genes. In addition, siderophore biosynthesis and ferric–siderophore receptor genes are regulated and are activated when iron is limited.

2.2.4 Siderophores in the Rhizosphere

In pseudomonads, as many as fifteen enzymes are involved in the synthesis of some pyoverdin siderophores, and a number of additional proteins are required for the transport of ferric–siderophore complexes and in the regulation of siderophore and receptor expression. The simplest explanation for why microbes have invested such a significant amount of their resources to synthesize and utilize siderophores would suggest that these resources are needed because iron is essential for survival. In addition, the ability to obtain iron efficiently in an iron limited environment and at the expense of other microbes provides a means for a bacterium to compete for limited resources in the rhizosphere.

Synthesis of siderophores enables microbes to scavenge environmental iron, but several factors contribute to the success of those siderophores in binding the iron. The amount of siderophore produced is an important factor. Because the binding relationship between siderophore and iron is stoichiometric, the more siderophore molecules available, the more iron that can be bound. In addition, the greater the affinity the siderophore has for iron and the faster the rate of association of the iron and siderophore, the more success the bacterium will have in obtaining iron. However, in order to bind iron, siderophores must lose protons. Therefore, to form a stable complex with iron is, in part, a function of its tendency to lose protons that in turn is influenced by rhizosphere pH. The association of siderophore and iron is thus reduced in acidic soils; however, ferric iron is reduced to a more soluble ferrous state in acidic conditions and therefore siderophore-mediated iron chelation is not as critical for iron acquisition under these conditions. The more protons that a siderophore has to lose, the more pH dependent it is.

In the rhizosphere, the capacity to produce large amounts of high affinity siderophores may not to be as important for bacterial competition and root colonization as the ability to utilize a variety of different ferric–siderophores. Thus,

the expression of relatively nonspecific receptors that can bind a range of ferric–siderophores or the production of several siderophore receptors with different binding specificities gives a bacterium a competitive edge on the plant surface.

The best competitors therefore are those that can utilize a broad range of siderophores and at the same time produce siderophores that few other microbes can use. Some pseudomonads produce outer membrane receptors that recognize a variety of ferric–siderophores and also secrete siderophores that most other bacteria are unable to bind and thus are particularly effective biocontrol agents. By the use of genetic engineering to extend the spectrum of siderophores that a PGPB can recognize, it may be possible to increase the capacity of these bacteria to facilitate plant growth.

Siderophores may also help to alleviate the stress imposed on plants by high soil levels of heavy metals. *Kluyvera ascorbata*, a siderophore-producing PGPB, was able to protect plants from heavy metal (nickel, lead, and zinc) toxicity. Moreover, a siderophore-overproducing mutant of this bacterium provided even greater protection, in three different plants (canola, Indian mustard, and tomato) cultivated in nickel contaminated soil. In this case, the siderophores of *K. ascorbata* are directly supplying iron to the contaminated plants, thereby relieving them of iron deficiency, a symptom of heavy metal toxicity.

2.3 Solubilizing Phosphorus

Plant growth requires a significant amount of phosphorus, a key component in both cell membranes and nucleic acids. Fortunately, most soils contain quite a lot of phosphorus (~400–1,200 mg/kg of soil). However, most of the phosphorus in soil is insoluble and is therefore not available to support plant growth. Thus, the amount of soluble phosphorus in many soils is ~1 mg/kg of soil. The insoluble phosphorus includes both inorganic and organic forms. The organic phosphorus in soil (typically ~30–50 % of the total) is mostly in the form of inositol hexaphosphate (phytate) that is the principal storage form of phosphorus in plants (Fig. 2.24). Phytate is not digestible by humans or non-ruminant animals. Moreover, phytate is not bioavailable to plants as plant roots generally produce only very low levels of phytases, the enzymes that break down phytate. Nevertheless, a number of different microorganisms (both bacteria and fungi) readily degrade phytate. In addition to phytate, some of the other forms of organic phosphate found in the soil include phosphomonoesters and phosphotriesters that can be broken down by various phosphatases.

The inorganic forms of phosphorus include minerals such as apatite. Apatite is really a group of phosphate minerals that includes hydroxyapatite, fluorapatite, and chloroapatite. Hydroxyapatite is also the major component of tooth enamel and bone mineral. Apatite is often ground into a powder and used as a fertilizer as a source of phosphorus. Plants and bacteria typically solubilize inorganic

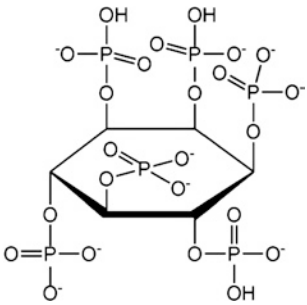

Fig. 2.24 The structure of phytate (phytic acid)

phosphorus through the production and secretion of small organic acids such as gluconic, citric, lactic, 2-ketogluconic, oxalic, tartaric, and acetic acid. These organic acids act as chelating agents that solubilize minerals that contain phosphorus, and in the process they sometimes solubilize some other nutrients at the same time. Unfortunately, a considerable fraction of the soluble inorganic phosphorus that is used by farmers as a chemical fertilizer is immobilized soon after it is applied. This means that much of this chemical phosphorus fertilizer that is applied is unavailable to plants and is therefore wasted and ultimately contributes to eutrophication (i.e., the run-off of nutrients from fields into bodies of water where these nutrients are likely to promote algal growth).

One simple way of testing whether a PGPB strain can solubilize inorganic phosphorus is to plate the bacterium on solid medium that contains inorganic phosphorus and, following growth of the bacterium, look for a halo or zone of clearance around the bacterial colony (Fig. 2.25). Such a zone of clearance indicates that the bacterium is synthesizing and secreting organic acids that solubilize that inorganic phosphorus. This simple plate assay may also serve to facilitate the isolation of genes involved in organic acid production.

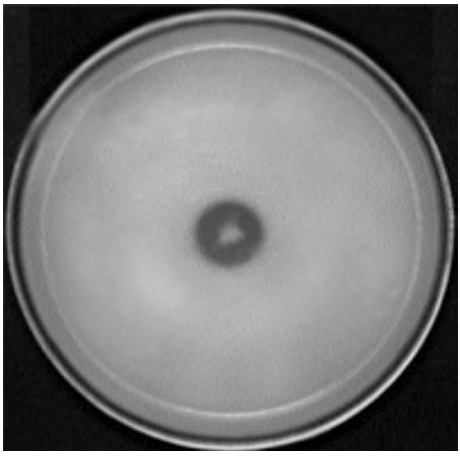

Fig. 2.25 Petri plate showing a zone of clearance around a inorganic phosphorus-solubilizing bacterium

Table 2.8 Growth of tobacco plants for 90 days in sterilized soil pots in the greenhouse with different treatments

Characteristic	Treatment		
	No added bacteria	Wild-type *B. mucilaginosus*	Transformed *B. mucilaginosus*
Plant height (cm)	17.4	21.4	24.7
Plant dry weight (mg)	1,297	1,685	1,870
Leaf P content (mg/g)	710	732	800

Transformed *Bacillus mucilaginosus* included a phytase gene from the fungus *Aspergillus fumigatus*

It is important to note that sometimes the same bacterial strain can naturally solubilize both organic and inorganic forms of phosphorus. This also means that it is possible to engineer more efficient strains of phosphorus-solubilizing bacteria. In one experiment, a phytase gene was isolated from the fungus *Aspergillus fumigatus,* placed under the control of a bacterial promoter and, with a transposon, inserted into the chromosomal DNA of *Bacillus mucilaginosus* that can dissolve inorganic phosphorus. When this transformed bacterium was tested in both greenhouse and field experiments, it was more efficient at providing phosphorus to tobacco plants than the non-transformed strain of this bacterium (Table 2.8). While, it is currently unlikely that any genetically engineered strain will be approved for widespread environmental dissemination, this is an important step in trying to overcome the inconsistent and disappointing results observed so far in the employment of naturally occurring phosphorus-solubilizing bacteria. Finally, it is possible that in nature, phosphorus solubilization is not preformed exclusively by naturally occurring phosphorus-solubilizing bacteria but rather includes a variety of soil fungi (such as mycorrhizae) as well.

Questions

1. What are cyanobacteria and how might they be useful as a biological fertilizer?
2. What are heterocysts?
3. How can some strains of cyanobacteria be genetically improved so they fix more nitrogen?
4. Why is the process of nitrogen fixation energy intensive?
5. How is the nitrogen fixation apparatus with a bacteroid protected against the inhibitory effects of oxygen?
6. How is bacteroid nitrogen fixation coordinated with plant photosynthesis?
7. How can nitrogenase activity be assayed biochemically?
8. Why is it unlikely that plants can be genetically engineered to fix nitrogen?
9. What is walking down the chromosome and how does it work?
10. How do NifA and NifL regulate nitrogenase expression?
11. How might glycogen synthase affect nitrogen fixation?
12. How would the presence of an active *Vitreoscilla* sp. hemoglobin gene affect nitrogen fixation?

13. How can the *nifH* promoter be manipulated to increase nitrogen fixation?
14. How might the inability of a *Rhizobium* strain to produce the poly-β-hydroxybutyrate affect nitrogen fixation?
15. How can nitrogenase be used to provide a sink for atmospheric carbon dioxide?
16. How are *nod* genes turned on?
17. How can a Rhizobium strain be selectively attracted to a host legume?
18. What are some of the major events that occur during nodulation?
19. How does modifying ethylene levels affect nodulation efficiency?
20. How does the presence of the enzyme hydrogenase affect nitrogen fixation?
21. How can the level of hydrogenase in a bacterium be increased?
22. What are bacterial siderophores and how do they affect plant growth?
23. How would you isolate siderophore biosynthesis genes from a newly isolated PGPB?
24. How do PGPB facilitate plant uptake of inorganic phosphate from the soil?
25. How do PGPB facilitate plant uptake of organic phosphate from the soil?

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