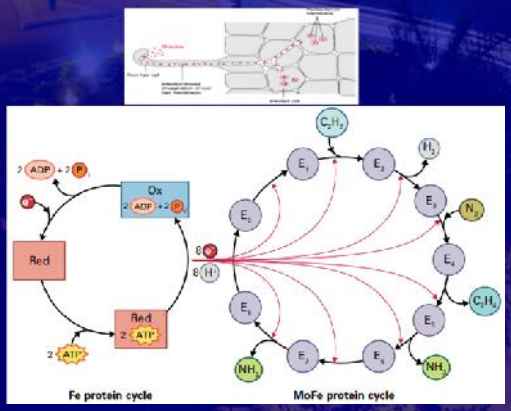
http://smtom.lecture.ub.ac.id/
Password:

https://syukur16tom.wordpress.com/
Password:

Lecture 09:
BIOLOGICAL N FIXATION



Biological nitrogen fixation (BNF) is the term used for a process in which nitrogen gas (N_2) from the atmosphere is incorporated into the tissue of certain plants.

LEARNING OUTCOMES

Students, after mastering materials of the present lecture, should be able

1. to explain the requirement of nitrogen nutrient in relation to biological nitrogen fixation (BNF).
2. to explain the reaction of N_2 reduction to NH_3 in in industrial system and BNF.
3. to explain nitrogenase and it components as the enzyme catalyzing N_2 reduction in BNF
4. to explain leghemoglobin responsible for the protection of nitrogenase from O_2 inactivation.
5. to explain the establishment of BNF including plant-microbe interaction and nodule development.

LECTURE OUTLINE

I. INRODUCTION
1. Nitrogen Requirement
2. Diazotrops

II. NITROGENASE
1. Nitrogenase Complex
2. MoFe Protein and Cofactor
3. Fe Protein
4. Leghemoglobin

III. SYMBIOTIC N FIXATION
1. Importance of N Fixation
2. N-Fixation Symbioses
3. Root Nodules
4. Legume-Rhizobia Interaction
5. Nodule Formation

I. INTRODUCTION

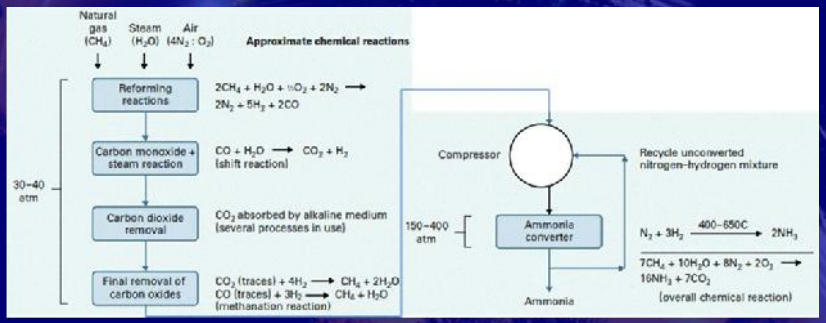
1. Nitrogen Requirement

- All plants need relatively large amounts of nitrogen (N) for proper growth and development.
  - About 22.2 kg N (48.3 kg urea) is required by a rice crop to produce a ton of grain (96,5 kg urea/ton grain or >500 kg urea/6 ton grain if N recovery of 50% is taken into account).
- In a closed ecological system, the nitrogen required for plant growth under natural condition is derived from the degradation of the biomass.
- In contrast to other plant nutrients (e.g., phosphate or sulfate), nitrogen (nitrate) cannot be delivered by the weathering of rocks.
- Nitrate in small amounts is generated by lightning and carried into the soil by rain water (in temperate areas about 5 kg N/ha per year).

- Due to the effects of civilization (e.g., car traffic, mass animal production, etc.), the amount of nitrate, other nitrous oxides and ammonia carried into the soil by rain can be in the range of 15 to 70 kg N/ha per year.
- Air (dry), by volume, contains 78.09% nitrogen (N_2) and other gases (20.95% O_2, 0.93% Ar, 0.04% CO_2, and other trace gases), but the N_2 cannot be used directly by eukaryotes.
- Nitrogen fertilizer and other fertilizers are crucial for agricultural production to compensate for those lost by the withdrawal of harvest products.
- Nitrogen fertilizer is produced from nitrogen and hydrogen by the **Haber-Bosch process** that produces ammonia used to synthesize urea or nitrate fertilizer.

$3H_2 + N_2 \rightarrow 2\ NH_3$ (ΔH-92.6 kJ/mol)

- The reaction of (**$3H_2 + N_2$**) requires a high activation energy due to the high bond energy of the N≡N triple bond, and has to be carried out at high pressures (30-400 atm) and high temperatures (400–500°C) that involves very high energy costs.



A flow scheme for the Haber Process

https://image.slidesharecdn.com/fakorede001421668haber-bosch-160401181116/95/haberbosch-process-3-638.jpg?cb=1461081720

2. Diazotrophs

- Some microbes called **diazotrophs**, which include many different eubacteria as well as some methanogenic archaea, are able to reduce the N_2 in the atmosphere to ammonia (NH_3) through a reaction catalysed by enzyme **nitrogenase** as follows:

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

- Unlike the Haber–Bosch process, such biological nitrogen fixation occurs at ambient temperatures and atmospheric pressure.
- Diazotrophs are represented by free-living microbes as well as a few species of bacteria that are able to establish symbiotic associations with plants.

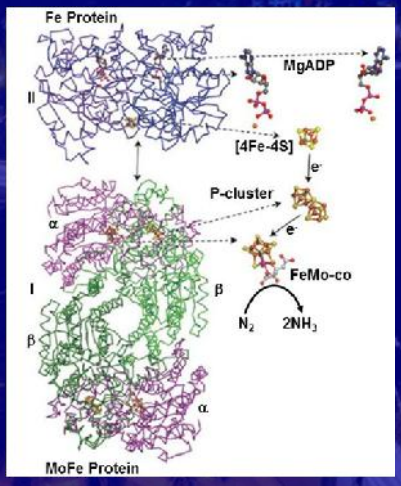
II. NITROGENEASE

1. Nitrogenase Complex

1. There are at least three classes of nitrogenase found in various nitrogen-fixing bacteria that differ in their metal composition:

   (i) molybdenum (Mo) nitrogenase, (ii) vanadium (V) nitrogenase, and (iii) iron-only (Fe) nitrogenase.
2. The best-studied nitrogenase is the **molybdenum** (Mo)-dependent enzyme (Fig.16.6).
3. Nitrogenase, dependent on Mo, is a complex of the MoFe protein (dinitrogenase, component I)- a hetero-tetramer, and the Fe protein (dinitrogenase reductase, component II)- a homodimer (two similar parts).
4. The reduction of N_2 by nitrogenase involves a complicated interplay between the component I and II.

FIGURE 16.6 Structures of **MoFe** (Component I, nitrogenase) and **Fe** (Component II, nitrogenase reductase) proteins of nitrogenase, and electron flow through the two enzymes.



The Fe protein is encoded by nifH, and it accepts electrons from a carrier, such as ferredoxin or flavodoxin. The identity of the carrier varies, depending on the biological system involved. The Fe protein, with net hydrolysis of ATP, transfers single electrons at very negative potential to P-clusters in the MoFe protein. The MoFe protein, an $\alpha_2\beta_2$ heterotetramer of subunits encoded by nifD and nifK, accepts electrons at the FeMo cofactor (FeMoco) and binds H^+ ions and N_2 molecules in a stepwise cycle, ultimately leading to the production of H_2 and ammonia.

2. MoFe Protein and Cofactor

- The protein part of the MoFe protein, the site of N_2 biinding and reduction, is a $(\alpha\beta)_2$ heterotetramer with each αβ dimer representing a functioning catalytic unit.
- Each MoFe protein is, therefore, composed of two catalytic units, with each unit having a site for associating with an Fe protein.
- Each αβ dimeric unit of the MoFe protein contains two unique metal cluster assemblies, or **cofactors**. One cofactor, called the FeMo cofactor (**FeMoco**) is composed of Mo, Fe, sulfur (S), carbide (C), and the organic acid ***R*-homocitrate** that are arranged in a complex metal cluster with an overall stoichiometry of [1Mo–7Fe–9S–1C–1homocitrate] (Fig. 16.7).

- FeMoco is the site of N_2 binding and reduction, but exactly where N_2 binds to this metal cluster during the reaction is not yet established.

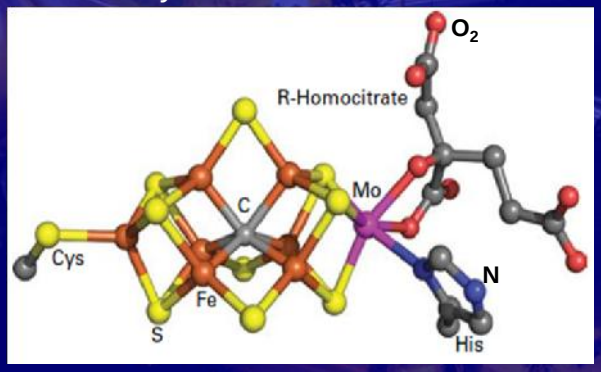


FIGURE 16.7 Molecular model of iron-molybdenum cofactor (**FeMo cofactor**). Carbon is shown in gray, iron in rust, sulfur in yellow, oxygen in red, nitrogen blue, and molybdenum in magenta. The atom at the center of FeMo-co is a carbide (C) atom C. The Mo-type of nitrogenase is present in all symbiotic bacteria, including Rhizobium and Bradyrhizobium.

- Each αβ dimer of the MoFe protein contains a second metal cluster called the P-cluster (Figs. 16.6 & 16.8). This cluster contains Fe and S with a stoichiometry of [8Fe-7S], arranged in an overall structure that can be viewed as a [4Fe–4S] cube bridged by an S at one corner to a [4Fe–3S] cube (Fig. 16.8).

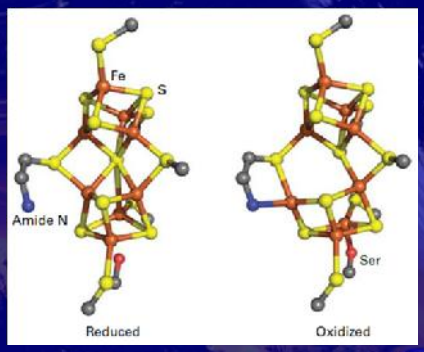


FIGURE 16.8 Structure of the P-clusters of the MoFe protein. Reduced and oxidized conformations are shown. Iron atoms are shown in rust, sulfur atoms in yellow.

- The complexity of the interactions between MoFe and Fe proteins is partly related to the transfer of only one electron at a time from Fe protein to the MoFe protein which is coupled to the hydrolysis of two MgATP molecules to yield two MgADP and two Pi.

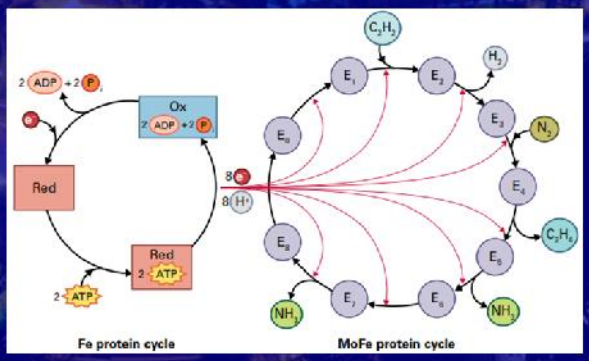
3. Fe Protein

- The Fe protein ,constructed from two identical protein subunits (¥$_2$), functions as a reductase to deliver electrons to the MoFe protein.
- The two γ_2 subunits are covalently linked to each other through a single [4Fe–4S] cube cluster that is bound by two cysteine amino acids from each subunit (Fig. 16.6).
- The [4Fe–4S] cluster is located at one end of the Fe protein.

- Each protein subunit also contains a nucleotide-binding site, which is located on the end of the protein away from the [4Fe–4S] cluster.
- The binding of two MgATP molecules, one to each subunit, changes the overall structure of the Fe protein.
- The reduction midpoint potential of the [4Fe–4S] cluster changes from –300 mV to –420 mV when MgATP binds.
- The initial transfer of a single electron from the [4Fe–4S] cluster of the Fe protein is activated by the hydrolysis of the two bound MgATP molecules.
- The reactions between the Fe protein and the MoFe protein can be viewed as two joined cycles (Fig. 16.9), with the Fe protein cycle driving the stepwise progression of the MoFe protein cycle.

FIGURE 16.9 The Fe and MoFe enzyme cycles.

E_n represents the redox state of the MoFe protein enzyme after it has accepted n electrons, and red arrows illustrate the transfer of a single electron and association of a proton.

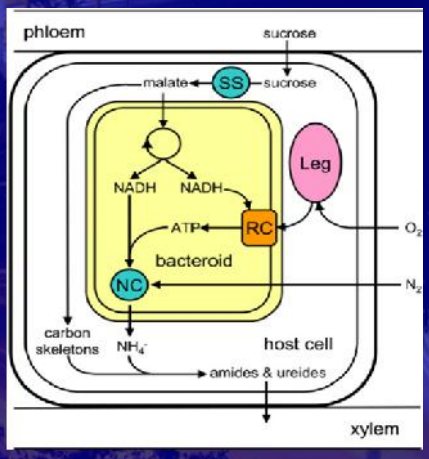


In the initial steps of the reaction, H_2 may evolve without productive binding of N_2, essentially wasting the ATP and reductant invested in creating these intermediates. Dinitrogen (N_2) can bind to the enzyme by displacing H_2 from either the E_3 or E_4 forms; thus, formation of molecular hydrogen is an obligatory step in the enzymatic reduction of N_2. Treatment of later complexes with acid releases ammonia, which suggests the N atoms in these complexes are associated with more than one H.

4. Leghemoglobin

- Nitrogen fixation consumes energy-rich compounds (ATP) and requires strong biological reductants (ferredoxin or flavodoxin).
- Because nitrogenase and some of the proteins that supply it with reductant are sensitive to oxygen, many nitrogen-fixing bacteria are anaerobes.
  - Anaerobic respiration is very inefficient in the oxidation of reduced carbon compounds, and large quantities of substrate are required for N_2 fixation.
  - The use of aerobic metabolism, very efficient in ATP production, must contend with the O_2 sensitivity of nitrogenase.
- In root nodules, the nitrogenase complex is protected from O_2 with the presence of leghemoglobin (Lb), a homolog of hemoglobin, binding O_2 that minimizes concentration of free O_2 in the nodules.

- Lb is an iron-containing protein and, like hemoglobin in red blood cells, reversibly binds O_2, and provides O_2 to the bacteroids for their intense respiration required to produce ATP for N_2 fixation.



A root nodule showing the exchange of C and N between the host and bacteriod. Leg, Leghemoglobin; RC, bacterial respiratory chain; NC, the nitrogenase complex; SS, Suc synthase.

https://www.researchgate.net/figure/26813384_fig2_Figure-2-A-root-nodule-showing-the-exchange-of-C-and-N-between-the-host-and-bacteriod

III. SYMBIOTIC N FIXATION

1. Importance of Biological N fixation

- In natural terrestrial ecosystems, 80–90% of the nitrogen available to plants originates from biological nitrogen fixation, and of that total, approximately 80% is produced in symbiotic associations.
- The benefits of nitrogen fixation, however, are not without cost. If all plant resources required to establish nodules, fix nitrogen, and transport the resulting ammonia throughout the plant are taken into account, symbiotic N_2 fixation consumes 12–17 g of carbohydrate per gram of nitrogen fixed.
- Predictably, legumes have mechanisms to suppress nodule formation and function if nitrate or ammonia is available as an alternate source of nitrogen.

2. N-Fixing Symbioses

- Hosts for nitrogen-fixing bacteria include fungi (lichens) and animals ranging from marine corals to terrestrial termites.
- In the case of vascular plants, three major groups of prokaryotes establish morphologically developed N_2-fixing symbioses.
- The first group of symbioses exists between cyanobacteria, such as Anabaena, and diverse plants, including cycads, ferns, liverworts, hornworts, and one angiosperm genus, Gunnera.
- These hosts elaborate specialized structures to accommodate the cyanobacteria. For example;
  - Gunnera is infected in glands at the base of leaf petioles,
  - Cycads produce specialized “coralloid“ roots, and

- Azolla, a water fern that associates symbiotically with the cyanobacterium Anabaena, harbors the bacteria in a leaf cavity. The Azolla–Anabaena pair is used as a cocrop with rice (Oryza sativa); it produces sufficient fixed nitrogen to allow continuous and sustainable rice cultivation.

- A second type of symbiosis occurs between members of a Gram-positive actinomycete genus (Frankia) and a diverse group of dicots from over 20 genera in eight or more families within the Rosid I lineage of dicotyledonous plants (Fig. 16.11).
- The hosts include trees or woody shrubs such as alder (Alnus), myrtle (Myrica), Casuarina, and Ceanothus.
- These symbiotic associations play a significant role in the nitrogen economies of forests and other natural ecosystems.

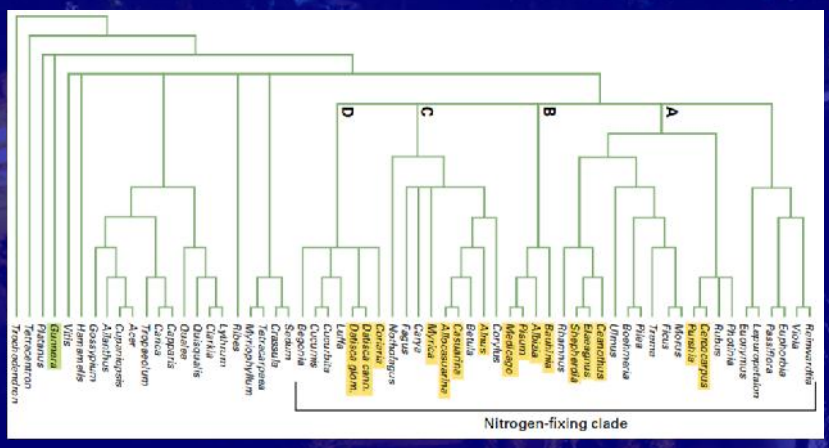


FIGURE 16.11 Phylogenetic analysis of 99 rbcL genes (encoding the large subunit of Rubisco) from genera representing the subclass Rosidae and additional angiosperm taxa. Taxa that engage in nodular nitrogen-fixing symbioses are highlighted in yellow. (Gunnera, which hosts cyanobacterial endosymbionts, is marked in green.) Note that a single clade includes all the nodule-forming genera included in the tree. Subclades A, C, and D include actinorhizal symbiotic hosts; the legume family (Fabaceae) falls into subclade B

- A third group of symbioses involves rhizobia, which are Gram-negative bacteria, that infect numerous legumes (*Fabaceae*) and one known nonlegume, Parasponia (*Ulmaceae*).
- These plants are also in the Rosid I lineage (Fig. 16.11). Rhizobial endosymbionts contribute nitrogen to commercially important seed legumes such as peanut (*Arachis hypogaea*), soybean (*Glycine max*), lentil (*Lens culinaris*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), cowpea (*Vigna unguiculata*), and forage crops such as clover (*Trifolium* spp.) and alfalfa (*Medicago sativa*).
- Rhizobial symbioses are also important in plants used for land stabilization and reclamation, and in natural environments.

3. Root Nodules

- In the presence of compatible rhizobial species, legume hosts also form root nodules (Fig. 16.12).
- Initially it was thought that the nodules of legumes were caused by a plant disease, until their function in N_2 fixation was recognized by Hermann Hellriegel (Germany) in 1888.

FIGURE 16.12 Photo of root nodules on pea (Pisum sativum). Source: Long, Stanford University, Palo Alto, CA; previously unpublished. Bacterial species

- The nodule systems establish environments conducive to N_2 fixation, especially the microaerobic conditions needed for bacteria to synthesize ATP and stabilize nitrogenase.
- Plant metabolism in the nodule generates organic acids, some of which are catabolized by the rhizobial symbionts to fix nitrogen.
- The bacteria release the resulting ammonia to the plant, which assimilates it into amides or ureides used to transfer fixed (reduced) N to the rest of the plant.
- Bacterial nitrogen fixation is most useful to the plant when it has no other nitrogen sources. Few rhizobia fix nitrogen outside the plant host.
- Nodule formation and function are subject to downregulation by host plants that are provided with nitrate or ammonia.

4. Legume-Rhizobia Interaction

- Accurate mutual recognition in legume-rhizobia systems is accomplished by exchange of biochemical signals.
- Molecules released by the host plant induce bacterial gene expression (Fig. 16.13) that leads to production of bacterial signal molecules and proteins that modify plant metabolism and development.
- Specific bacterial genes are involved in the sequential stages of symbiosis.
  - nod genes are rhizobial genes responsible for early nodule formation, and expressed on the basis of a chemical signal from the plant (the inducer) and on a bacterial transcription activator, NodD (Fig. 16.13B).
- The plant signals that induce nod gene expression are commonly flavonoids (Fig. 16.13C), and their diverse structures provide initial recognition for plant–bacteria communication.

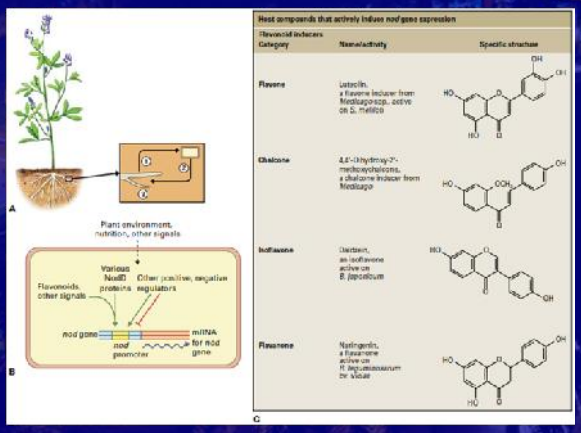


FIGURE 16.13 Signals in early nodule development. (A) Plant and bacteria exchange molecular signals. In many cases, step 1 is plant production of a root-derived flavonoid, and step 2 is recognition of the flavonoid and production of a bacterial Nod factor, which triggers responses in the plant (step 3). (B) In the bacterium, the transcriptional activator NodD interacts with a plant inducer and the transcriptional machinery of the bacterium to induce expression of bacterial nod genes. The circuits for nod gene activation may also respond to environmental cues and as yet unknown signals from the plant. (C) Flavonoids, the prominent inducers of rhizobial nod gene expression. The plant compounds that most actively induce expression of nodulation genes vary among different plant-symbiont systems. A bacterium's nodD genotype is a primary determinant of the preferred inducer structure.

- Symbiotic rhizobia also produce various molecular signals that trigger nodulation-related changes in the host plant, including oligo- and polysaccharides and proteins.
- Many rhizobial nod genes, for example, encode enzymes that direct the synthesis of Nod factors, bacterial products that act as nodule morphogens.
- Nod factors are lipooligosaccharides, fatty acid derivatized oligomers of chitin [β-1,4-linked N-acetylglucosamine (GlcNAc)] (Fig. 16.13D).
- The core Nod factor structure is a chitin backbone of three to five GlcNAc residues with an Nacyl substitution on the nonreducing end GlcNAc residue.
- This basic structure is constructed by three proteins common to all Nod factor-producing strains: NodA, NodB, and NodC.

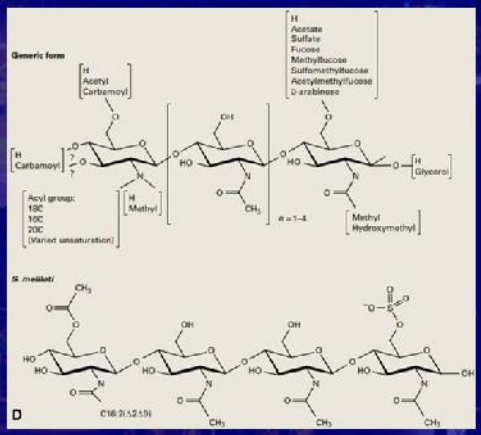


FIGURE 16.13 (Continued) (D) Structures of Nod factors, the N-acylated chito-oligosaccharides produced by bacteria. Nod factors are synthesized by enzymes encoded in the bacterial nod genes (B) and exported from the bacteria to the plant (step 2 in A). Plants respond to Nod factors with changes in ion flux, calcium spiking, morphogenesis, and transcription (step 3 in A). Top: all known Nod factors have a linear backbone of β-1,4-linked N-acetylglucosamine. Modifications to the reducing and nonreducing ends differ according to bacterial strain or species. The Nod factor modifying groups determine which host plant(s) will display nodulation-like reactions to the factor. Bottom: the Nod factor synthesized by Sinorhizobium meliloti and active on Medicago species. The 6-O-sulfonyl modification on the reducing end residue (right) and the modifications on the nonreducing end (6-O-acetyl and N-acyl) affect activity and host specificity of the molecule.

- Enzymes made by nod genes may also form or modify other compounds that play a role in symbiosis, such as lipids and carbohydrates.
- Additional types of specific extracellular polysaccharides are required to invade many host plants.
  - For example, *S. meliloti* mutants with defects in the structure or processing of extracellular polysaccharides (**EPSs**) are unable to infect Medicago: infection thread growth after infection by these mutants is feeble and aborts early.
- Prominent rhizobial extracellular carbohydrates include loosely associated EPSs (e.g., **succinoglycan**; Fig. 16.14A); **lipopolysaccharides** that extend from the lipid-A anchor in the bacterial outer membrane; **K-antigens**, unusual EPSs that contain keto-deoxyoctanoic acid; **neutral cyclic beta-glucans**; and **cellulose**.

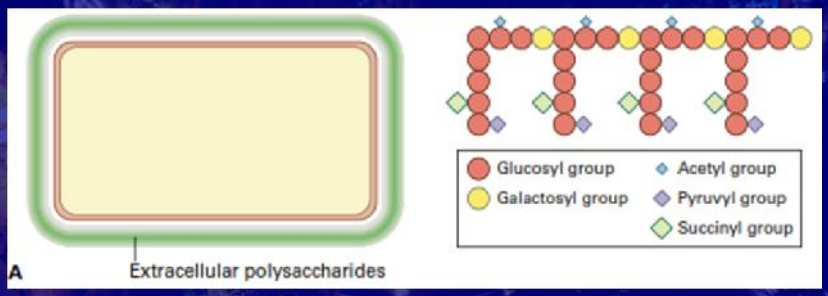


FIGURE 16.14 Extracellular molecules are important for rhizobial infection of some plant hosts. (A) Some rhizobial species, here exemplified by *Sinorhizobium meliloti*, produce extracellular polysaccharides (**EPS**), which are required for host invasion. Left: schematic of Gram-negative bacterium with capsular and exopolysaccharides exterior to the outer membrane. Right: structure of the well-studied EPS-I (succinoglycan) of *S. meliloti*. The repeating unit of the EPS is an eight-sugar branched oligomer, consisting of a backbone of four residues [→4)β-Glc-(1→4)β-Glc-(1→4)β-Glc-(1→3)β-Gal-(1→] and a four-residue side chain of mixed β1→3 and β1→6 Glc. The backbone and side chain are modified by acidic derivatization. The EPS contains hundreds of these eight-residue repeats, of which four are shown here.

- The roles played by various EPSs in symbiosis may differ from one host-rhizobial symbiotic pair to another.
- Finally, in some broad host-range bacteria, **Type III/Type IV secretion systems** appear to play a major role in determining which plants can be nodulated (Fig. 16.14B).

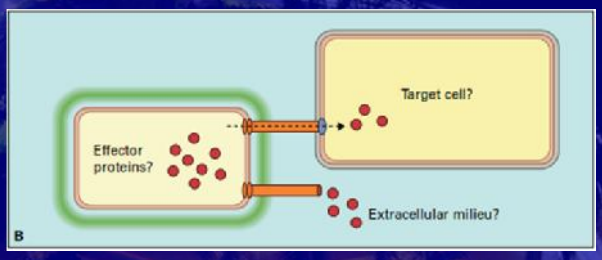
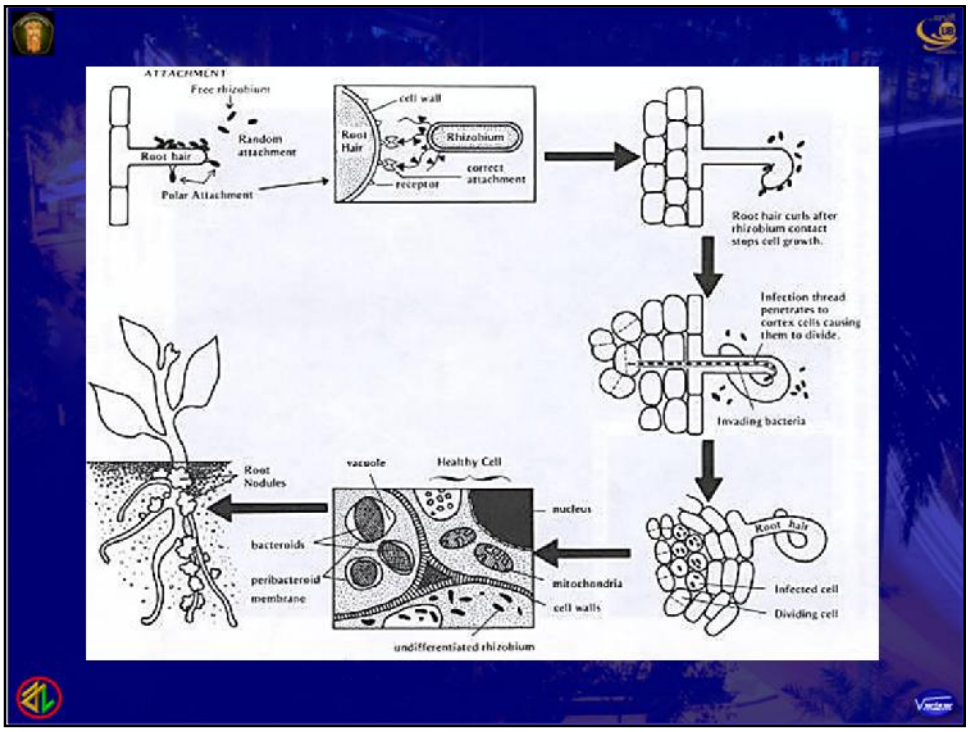


Figure 16.14B. In some broad host-range rhizobial strains such as Sinorhizobium fredii NGR234 and Mesorhizobium loti, Type III or IV secretion systems are required for symbiosis on some host plants. The secretion systems export proteins; in principle, these may be introduced into other bacterial cells, into plant cells, or into the medium.

5. Nodule Formation

- In many cases, bacterial infection begins in the root hair initiation segment of the root.
  - Cellular and molecular assays reveal multiple responses and altered root hair function following exposure to Nod factor (Table 16.4).
  - These responses, which include altered ion fluxes and **Ca^{2+} spiking**, likely signal developmental changes in the root hair, such as its growth into the curled structure known as the "shepherd's crook," which traps one or more bacteria against the developing cell wall (Fig. 16.15A).
- Over a period of hours to days, this wall deforms and extends into the root hair, leading to a tube-like structure known as the **infection thread**.
- As rhizobia grow and divide and the adjacent plasma membrane and infection thread wall expand, the bacteria push farther into the root (Fig. 16.15Bi).



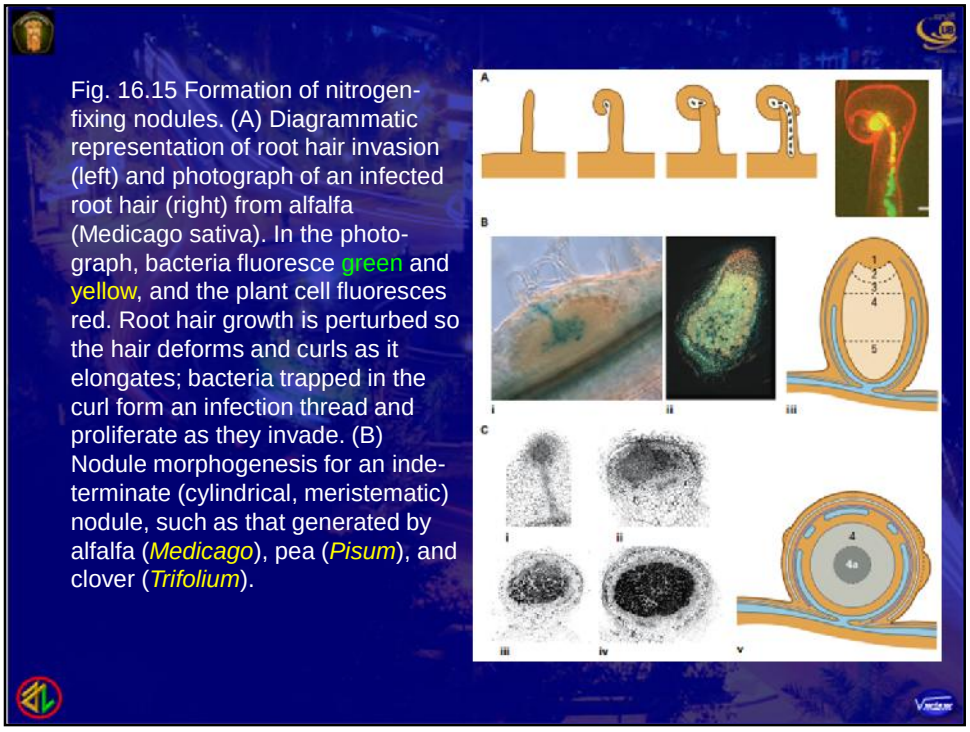
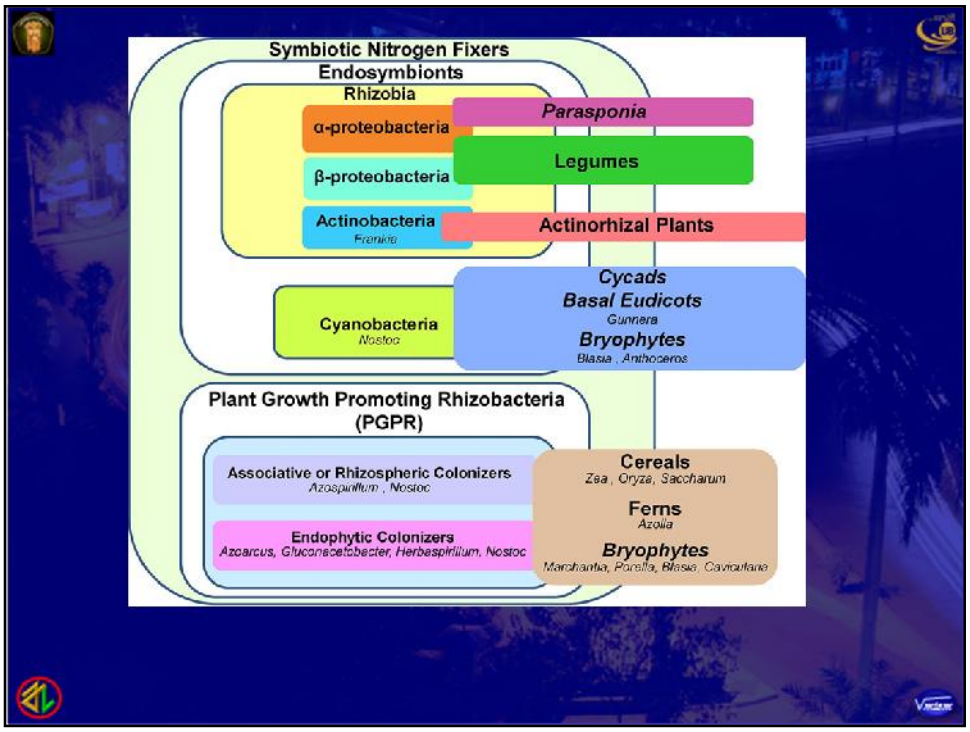
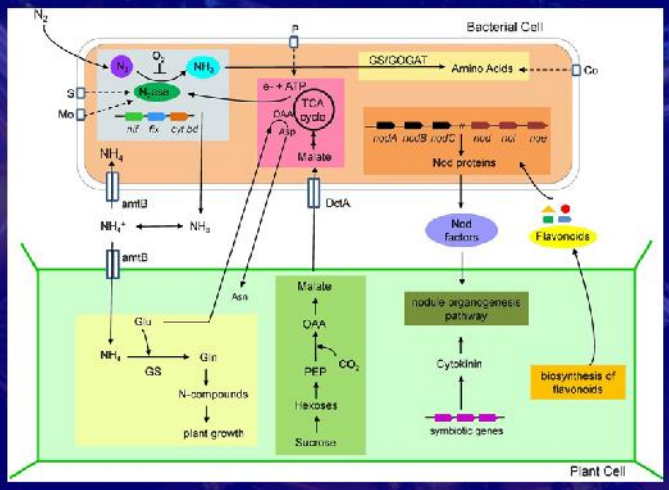


Fig. 16.15 Formation of nitrogen-fixing nodules. (A) Diagrammatic representation of root hair invasion (left) and photograph of an infected root hair (right) from alfalfa (Medicago sativa). In the photo-graph, bacteria fluoresce green and yellow, and the plant cell fluoresces red. Root hair growth is perturbed so the hair deforms and curls as it elongates; bacteria trapped in the curl form an infection thread and proliferate as they invade. (B) Nodule morphogenesis for an inde-terminate (cylindrical, meristematic) nodule, such as that generated by alfalfa (*Medicago*), pea (*Pisum*), and clover (*Trifolium*).

The first cell divisions occur in the inner cortical layer of the root and continue as the infection thread penetrates (i); bacteria in the infection thread are stained blue from a LacZ marker. A nodule meristem forms and persists so that newly divided, uninfected plant cells are continuously formed at the distal (from the root) nodule tip. An elongated nodule, formed by activity of a persistent meristerm, has zones of uninfected, infected, and senescent cells along a distal to proximal axis of the nodule. The nodule is stained with DAPI (labeling DNA and cell wall) and acridine orange (labeling RNA orange and DNA green) (ii). Numbers in diagram (iii) correspond to the following structures: 1, nodule meristem; 2, infection thread growth and cell penetration; 3, expanding infected cells; 4, mature bacteroid-containing tissue; 5, senescent bacteroid-containing tissue. (C) Nodule morphogenesis for a determinate (spherical) nodule, such as that produced by soybean (Glycine), trefoil (Lotus), and bean (Phaseolus). (i) Initial cell divisions are observed in the root outer cortex, followed by invasion of bacteria into plant cells; (ii–iv) many rounds of host and bacterial cell division give rise to zones of infected and uninfected cells in a spherical, stable nodule. Bars = 250 μm. (v) Schematic diagram of the zones of the fully formed determinate nodule: 4, mature bacteroid-containing tissue; 4(a), zone where senescence starts in determinate nodules. Mature determinate nodules lack meristematic tissues. Source: (A) Gage; (B) Long & Haney; © ASM press; (C) Long & Dudley Dudley © Springer citation Planta volume.





the exchange of nutrients between the two partners (a plant and a nitrogen-fixing microorganism). Rhizobia induce the formation of nodules on legumes using either Nod factor-dependent or Nod factor-independent processes. In the Nod factor-dependent strategy, plants release signals, such as flavonoids, that are perceived by compatible bacteria in the rhizosphere. Source: Mus et al. (2016)

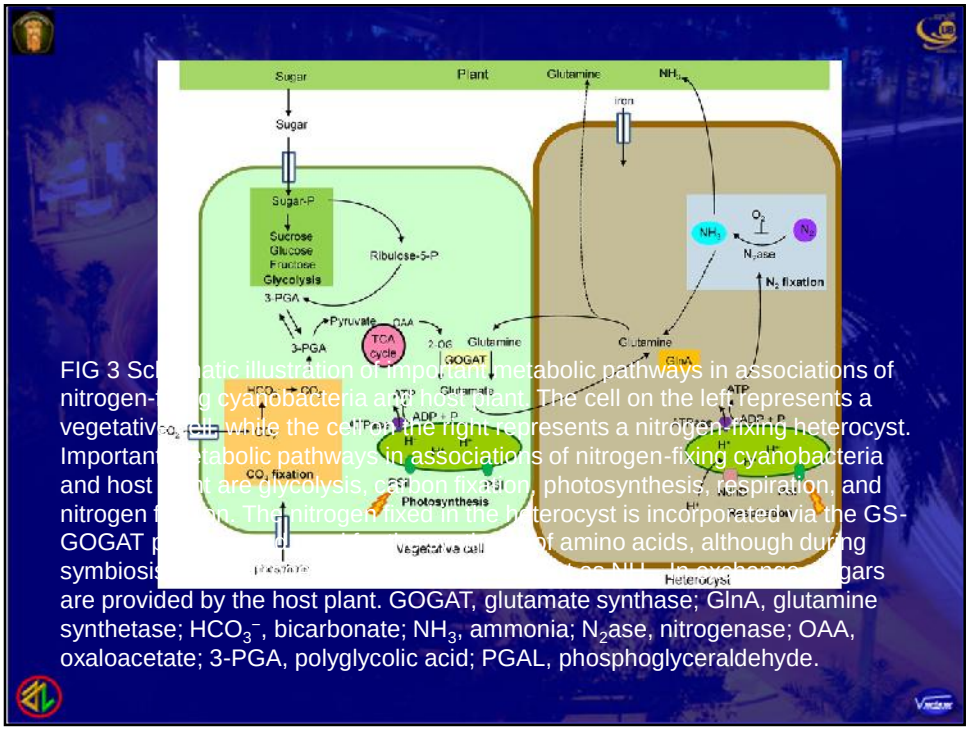


FIG 3 Sch[illegible]matic illustration of important metabolic pathways in associations of nitrogen-[illegible]g cyanobacteria and host plant. The cell on the left represents a vegetative cell, while the cell on the right represents a nitrogen-fixing heterocyst. Important metabolic pathways in associations of nitrogen-fixing cyanobacteria and host plant are glycolysis, carbon fixation, photosynthesis, respiration, and nitrogen fixation. The nitrogen fixed in the heterocyst is incorporated via the GS-GOGAT p[illegible] of amino acids, although during symbiosis[illegible] as NH_4. In exchange, sugars are provided by the host plant. GOGAT, glutamate synthase; GlnA, glutamine synthetase; HCO_3^-, bicarbonate; NH_3, ammonia; N_2ase, nitrogenase; OAA, oxaloacetate; 3-PGA, polyglycolic acid; PGAL, phosphoglyceraldehyde.