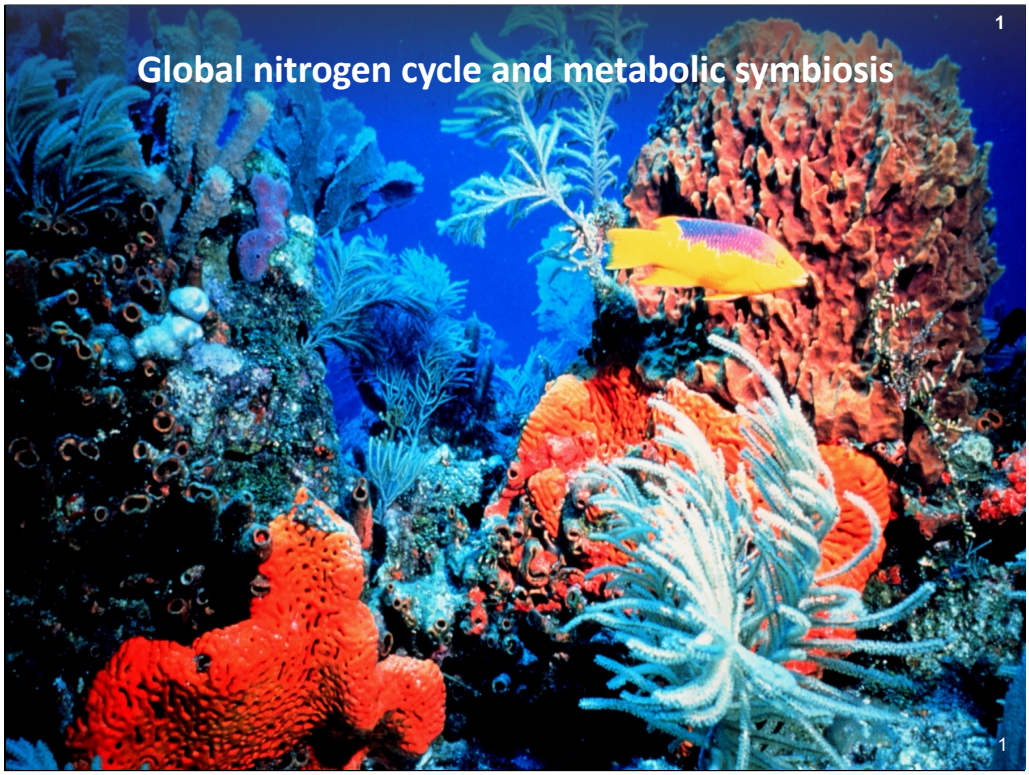
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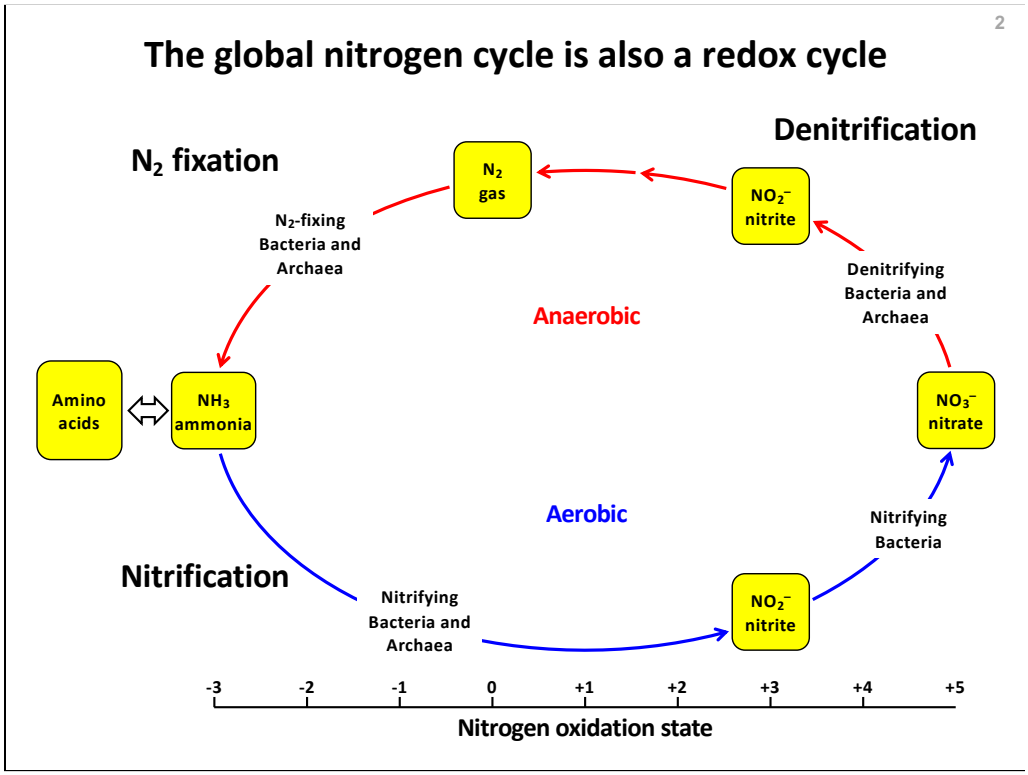
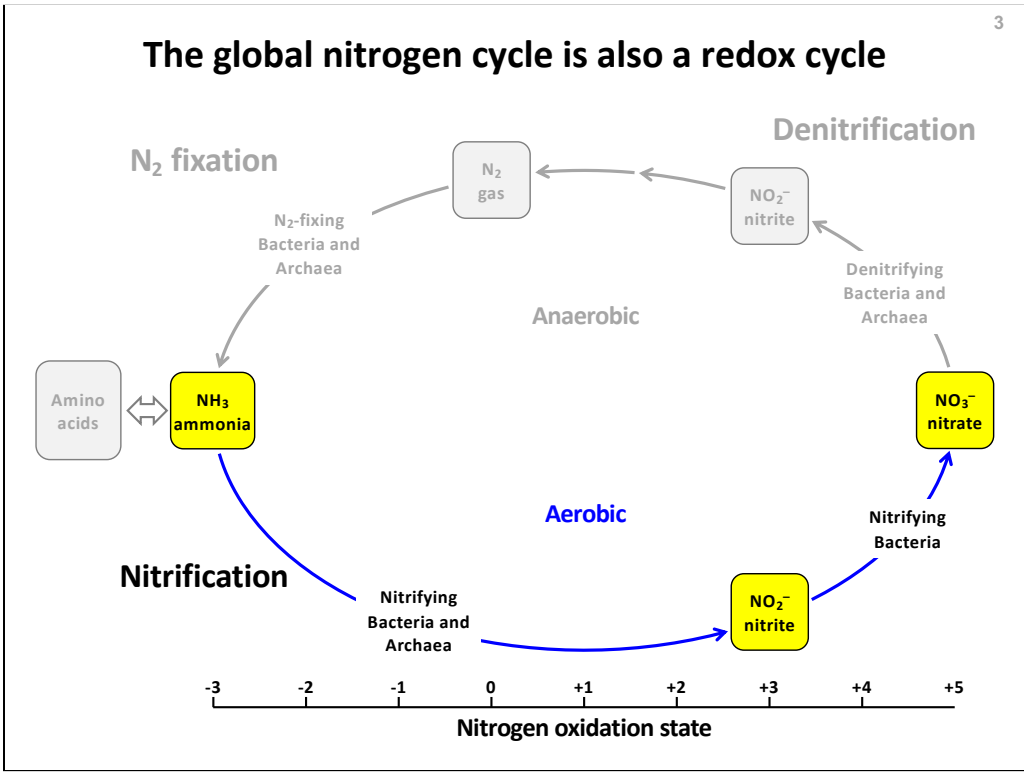
Global nitrogen cycle and metabolic symbiosis
1



SOURCE: *Lehninger Principles of Biochemistry* (p. 852), published by W.H. Freeman & Co., New York, NY © 2008.

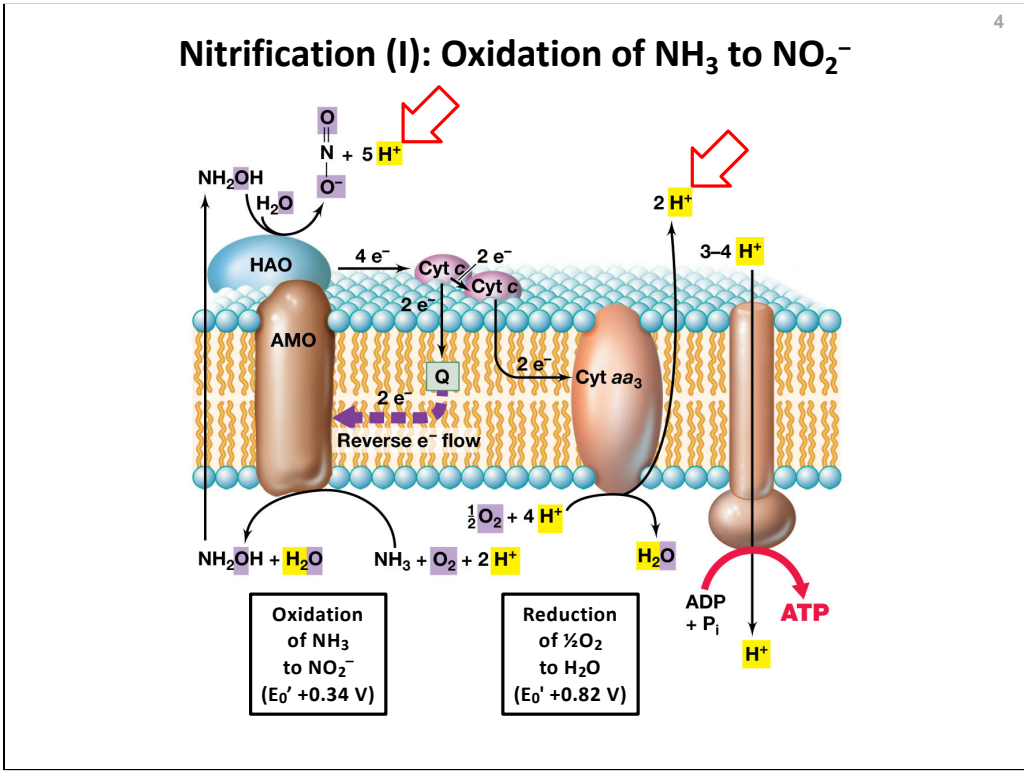
Figure 22.1. The nitrogen cycle. The total amount of nitrogen fixed annually in the biosphere exceeds 100,000,000,000 (10^{11}) kg. Reactions with blue arrows occur in aerobic environments. Reactions with red arrows occur in anaerobic environments. The redox states of the various nitrogen species are depicted at the bottom of the figure.



SOURCE: *Lehninger Principles of Biochemistry* (p. 852), published by W.H. Freeman & Co., New York, NY © 2008.

Figure 22.1. The nitrogen cycle. The total amount of nitrogen fixed annually in the biosphere exceeds 100,000,000,000 (10^{11}) kg. Reactions with blue arrows occur in aerobic environments. The redox states of the various nitrogen species are depicted at the bottom of the figure.

Oxygen almost always has an oxidation number of -2, with a few exceptions. These are: any compound where oxygen is bound to itself, like peroxides or superoxides, and any compound where oxygen is bound to fluorine. The NO_2^- ion (nitrite) has a charge of -1. The oxygens carry a total oxidation number of -4. To get to -1, N has to be -1 -(-4) = +3. The oxidation number for NO_3^- (nitrate) is -1. This is because oxygen always has an oxidation number of -2. The three oxygen atoms have a combined oxidation of -6, corresponding to their electromagnetic charge and the lone nitrogen has a charge, or oxidation number, of +5. Combined together, the -6 charge of the oxygen atoms and the +5 charge of the nitrogen produce a molecule with a net charge of -1.



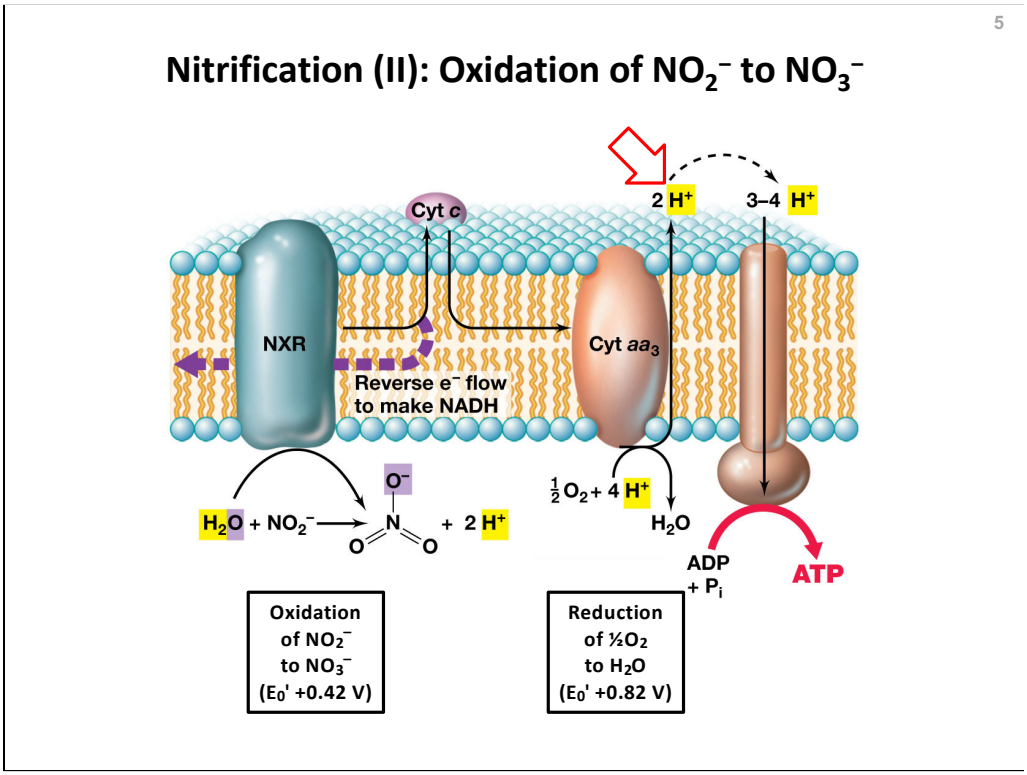
SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019.

Figure 14.32. Oxidation of NH_3 (**ammonia**) to NO_2^- (**nitrite**) and electron flow in NH_3 -oxidizing bacteria. The bioenergetics of nitrification is based on the same principles that govern other chemolithotrophic reactions: electrons from reduced inorganic substrates (in this case, reduced nitrogen compounds) enter an electron transport chain and electron flow establishes a proton motive force that drives ATP synthesis.

Nitrifying bacteria are faced with bioenergetic problems similar to those of most other chemolithotrophs. The E_0' of the NO_2^- /NH_3 couple (the first step in the oxidation of NH_3) is high, +0.34 V. The E_0' of the NO_3^- /NO_2^- couple is even higher, about +0.42 V. These relatively high reduction potentials force nitrifying bacteria to donate electrons to rather high potential acceptors in their electron transport chains, thus limiting the extent of electron transport and the available energy/ATP yield.

Several key enzymes participate in the oxidation of reduced nitrogen compounds. In NH_3 -oxidizing bacteria, NH_3 is oxidized by **ammonia mono-oxygenase** producing NH_2OH (**hydroxylamine**) and H_2O. A second key enzyme, **hydroxylamine oxido-reductase**, then oxidizes NH_2OH to NO_2^- (**nitrite**), removing four electrons in the process. In the reaction carried out by ammonia monooxygenase ($NH_3 + O_2 + 2\ H^+ + 2\ e^- \rightarrow NH_2OH + H_2O$), there is a need for two exogenously supplied electrons plus two protons to reduce one molecule of O_2 to water. These electrons originate from the oxidation of hydroxylamine and are supplied to ammonia mono-oxygenase from hydroxylamine oxido-reductase via cytochrome *c* and ubiquinone. Thus, for every four electrons generated from the oxidation of NH_3 to NO_2^-, only two actually reach the terminal oxidase (cytochrome aa_3) and can yield energy.

Abbreviations: Cyt *c*, cytochrome c; AMO, ammonia mono-oxygenase; HAO, hydroxylamine oxido-reductase; Q, ubiquinone.



SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019.

Figure 14.33. Oxidation of NO_2^- (**nitrite**) to NO_3^- (**nitrate**) by nitrifying bacteria. The reactants and products of this reaction series are highlighted to follow the reaction. NO_2^--oxidizing bacteria employ the enzyme **nitrite oxido-reductase** to oxidize NO_2^- to NO_3^-, with electrons traveling a very short electron transport chain to the terminal oxidase because of the high reduction potential (E_0' = + 0.42 V) of the NO_3^- /NO_2^- redox couple. Cytochromes of the *a* and *c* types are present in the electron transport chain of nitrite oxidizers, and the activity of **cytochromes *aa*$_3$** generates a proton motive force (PMF). Only small amounts of energy are available in this reaction. Thus, growth yields of nitrifying bacteria (grams of cells produced per mole of substrate oxidized) are low.

Abbreviations: NXR, nitrite oxido-reductase; Cyt *c*, cytochrome *c*; Cyt *aa*$_3$, cytochrome *aa*$_3$.

6

Humans, ammonia-oxidizing bacteria, and nitrite-oxidizing bacteria all use O_2 as the electron acceptor ($\frac{1}{2}O_2/H_2O$ with E_0' +0.82 V)) with NADH, NH_3, or NO_2^- as the electron donor:

A. Chemo-hetero-trophs (humans): $NAD^+/NADH$ (E_0' –0.32 V)

B. Ammonia-oxidizing bacteria: NO_2^-/NH_3 (E_0' +0.34 V)

C. Nitrite-oxidizing bacteria: NO_3^-/NO_2^- (E_0' +0.42 V)

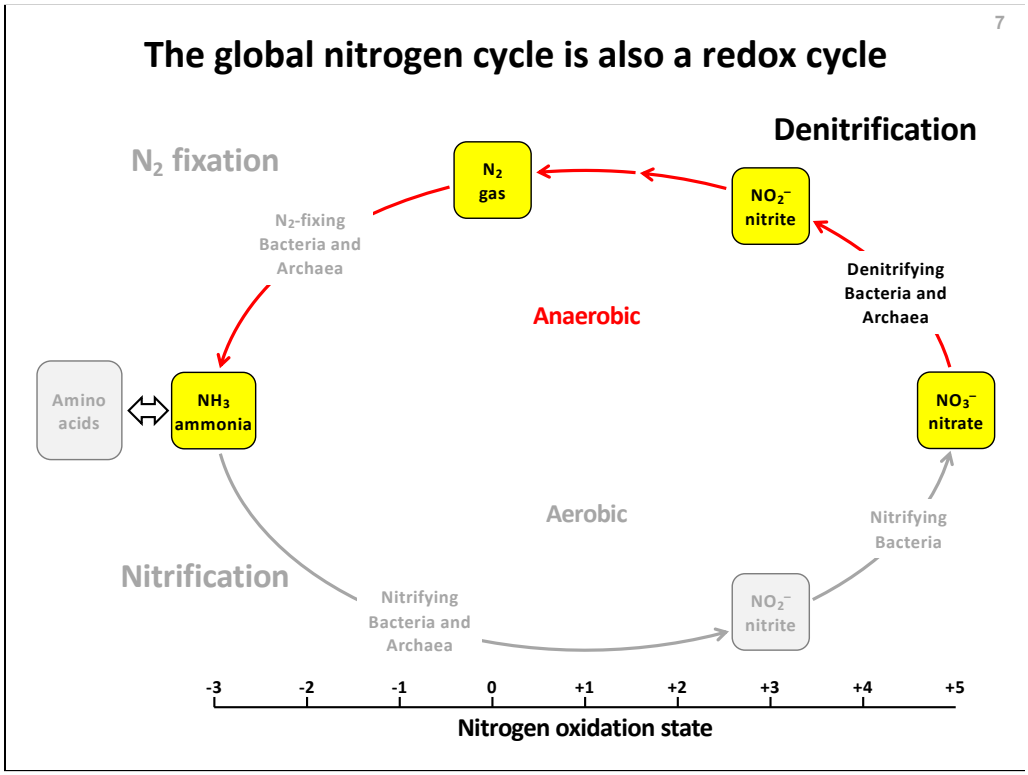
Which achieves the highest energy yield?

A. Chemo-hetero-trophs (humans).

B. Ammonia-oxidizing bacteria.

C. Nitrite-oxidizing bacteria.

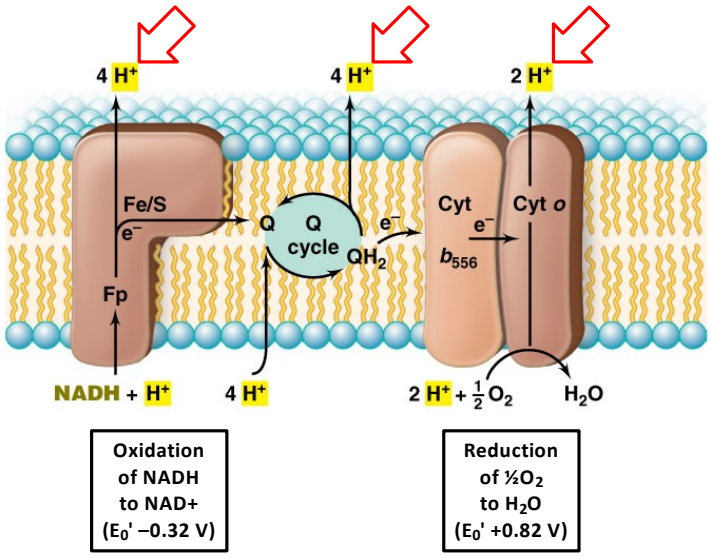
Answer: (A)



SOURCE: *Lehninger Principles of Biochemistry* (p. 852), published by W.H. Freeman & Co., New York, NY © 2008.

Figure 22.1. The nitrogen cycle. The total amount of nitrogen fixed annually in the biosphere exceeds 100,000,000,000 (10^{11}) kg. Reactions with blue arrows occur in aerobic environments. Reactions with red arrows occur in anaerobic environments. The redox states of the various nitrogen species are depicted at the bottom of the figure.

Aerobic respiration with O_2 as electron acceptor

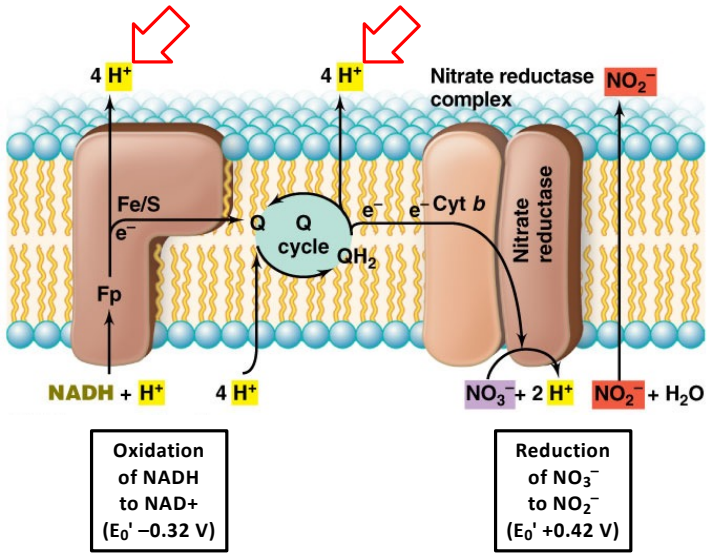


SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019.

Figure 14.36(a). Aerobic respiration when molecular oxygen (O_2) is the terminal electron acceptor and NADH is the electron donor. Under high-O_2 conditions, the sequence of carriers is Cyt b_{556} → Cyt o → O_2. However, under low-O_2 conditions (not shown), the sequence is Cyt b_{568} → Cyt d → O_2.

Abbreviations: Fp, flavoprotein; Fe-S, iron-sulfur protein; Q, ubiquinone; Cyt b_{556},, cytochrome b_{556}; Cyt o, cytochrome o.

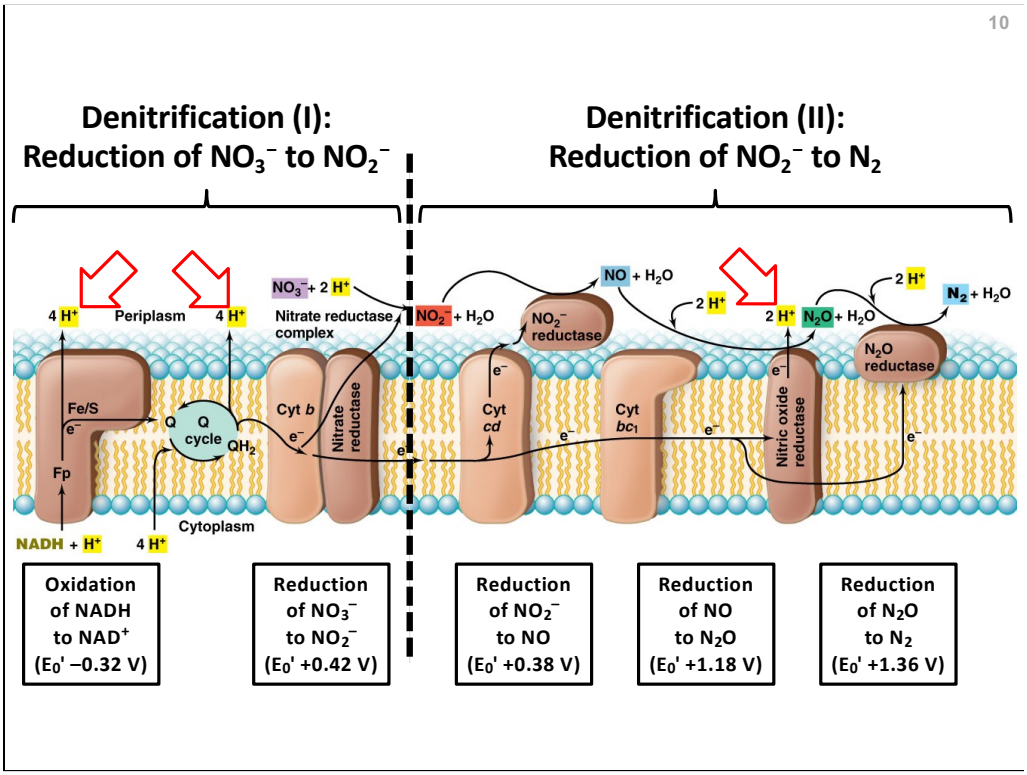
Anaerobic respiration with NO_3^- as electron acceptor



SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019.

Figure 14.36(b). Denitrification (I) is anaerobic respiration with NO_3^- (nitrate) as the terminal electron acceptor and NADH as the electron donor. Note how more protons are translocated per two electrons during electron transport reactions taking place aerobically with O_2 as the terminal electron acceptor (previous slide) rather than anaerobically with NO_3^- as the electron acceptor (this slide), because the aerobic terminal reductase (Cyt o) can pump two additional protons (previous slide), which the anerobic terminal reductase (nitrate reductase) cannot.

Abbreviations: NO_3^-, nitrate; NO_2^-, nitrite; Fp, flavoprotein; Fe-S, iron-sulfur protein; Q, ubiquinone; Cyt b, cytochrome b.

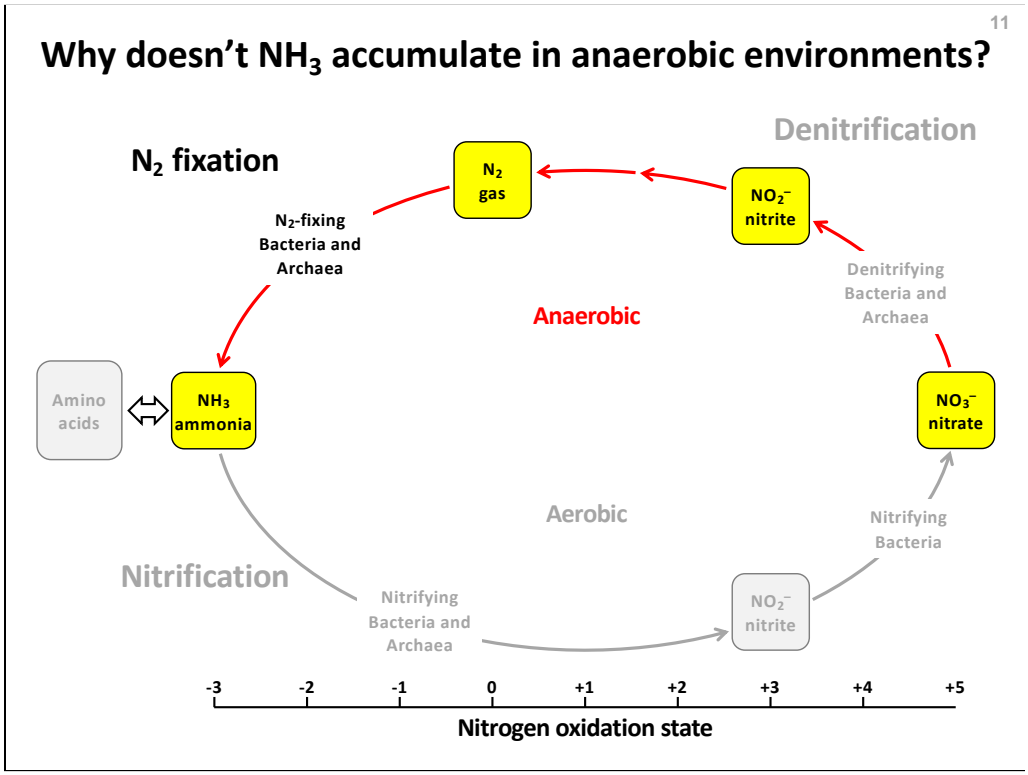


SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019.

Figure 14.36(c). Denitrification (I) and (II) is the main route by which gaseous dinitrogen (N_2) is formed biologically by sequential reduction of nitrogen oxide compounds. During electron transport, a proton motive force is established and ATPase functions to produce ATP in the usual fashion. Note that Denitrification (I) on this slide is the same as Denitrification (I) on the previous slide; it is shown again here to illustrate the transmission of electrons from Denitrification (I) to Denitrification (II) .

Enzymes: **Nitrate reductase** reduces NO_3^- (nitrate) to NO_2^- (nitrite). **Nitrite reductase** reduces NO_2^- (nitrite) to NO (nitric oxide). **Nitric oxide reductase** reduces NO (nitric oxide) to N_2O (nitrous oxide). **Nitrous oxide reductase** reduces N_2O (nitrous oxide) to N_2 (dinitrogen). Nitrate reductase and nitric oxide reductase are integral membrane proteins. Nitrite reductase and nitrous oxide reductase are periplasmic enzymes. All enzymes of the pathway are coordinately regulated (i.e., repressed) by O_2. In addition to anoxic conditions, NO_3^- (nitrate) must also be present before these enzymes are fully expressed.

Abbreviations: NO_3^-, nitrate; NO_2^-, nitrite; NO, nitric oxide; N_2O, nitrous oxide; N_2, dinitrogen; Fp, flavoprotein; Fe-S, iron-sulfur protein; Q, ubiquinone; Cyt b, cytochrome b; Cyt cd, cytochrome cd; Cyt bc_1, cytochrome bc_1.



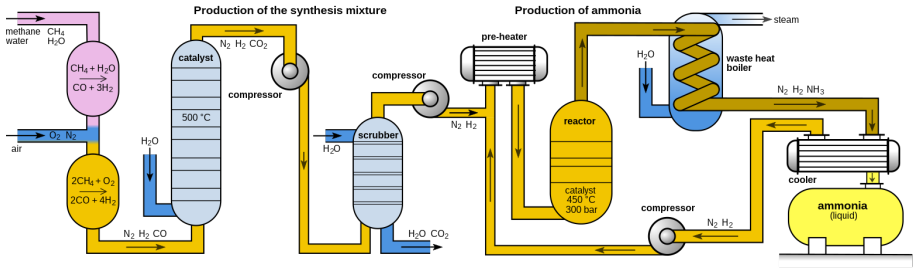
SOURCE: *Lehninger Principles of Biochemistry* (p. 852), published by W.H. Freeman & Co., New York, NY © 2008.

Figure 22.1. The nitrogen cycle. The total amount of nitrogen fixed annually in the biosphere exceeds 100,000,000,000 (10^{11}) kg. Reactions with blue arrows occur in aerobic environments. Reactions with red arrows occur in anaerobic environments. The redox states of the various nitrogen species are depicted at the bottom of the figure.

Industrial nitrogen fixation: Haber-Bosch process (1910)

$$N_2 + 3\ H_2 \rightarrow 2\ NH_3$$

- High temperature (500°C)
- High pressure (300 atm)
- Continuous removal of product



Source: https://people.idsia.ch/~juergen/haberbosch.html

Source: https://phys.org/news/2011-11-closer-soluble-solution-haber-bocsh.html

Fritz Haber was awarded the Nobel Prize in 1918 for his work on non-biotic NH_3 (ammonia) synthesis. The process he developed – now known as the “Haber Process” or the “Haber-Bosch Process” – fixes nitrogen from the air to make ammonia, which can be used to make synthetic fertilisers.

The Haber-Bosch process has often been called the most important invention of the 20th century, as it "detonated the population explosion," driving the world's population from about 1.6 billion in 1900 to more than 8.2 billion today.

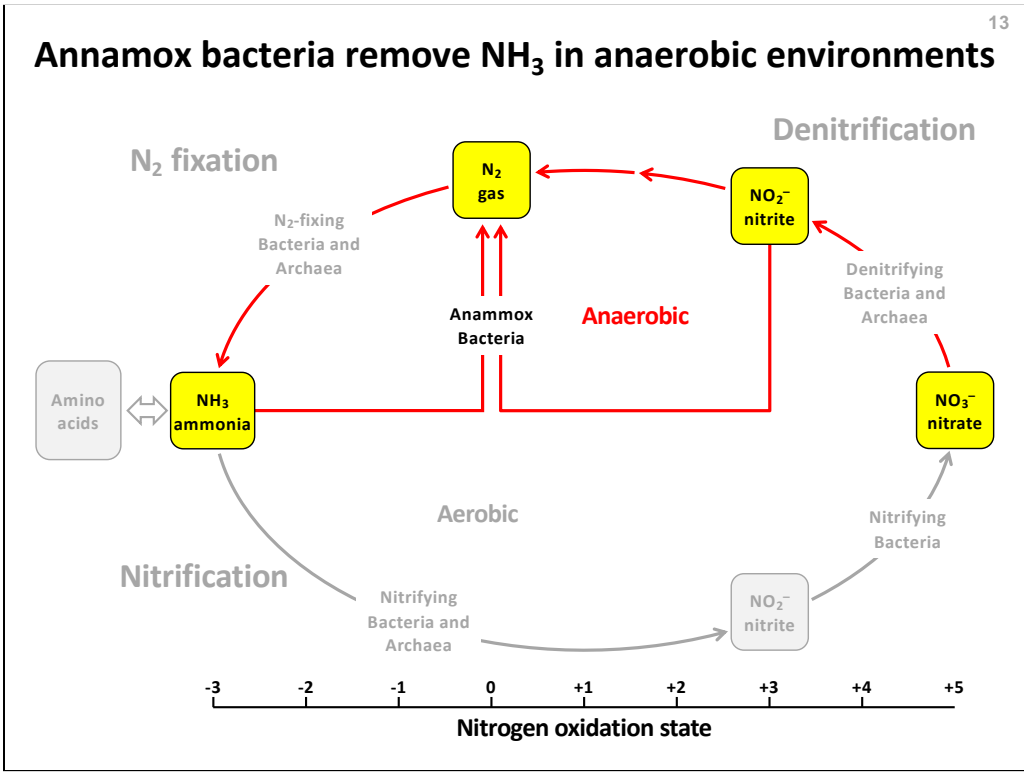


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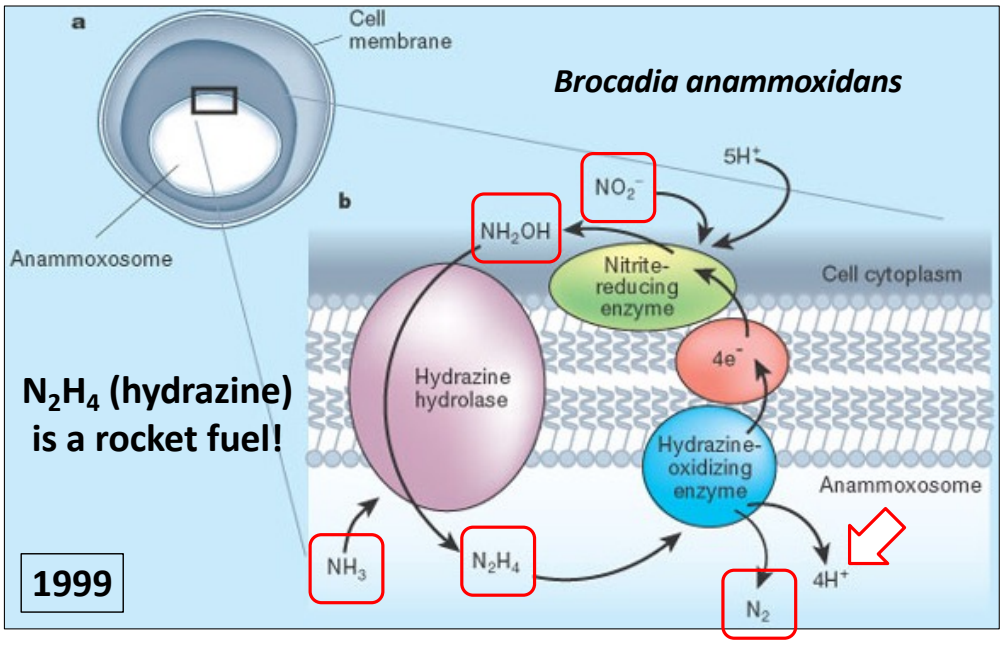
Figure 22.1. The nitrogen cycle. The total amount of nitrogen fixed annually in the biosphere exceeds 100,000,000,000 (10^{11}) kg. Reactions with red arrows occur in anaerobic environments. The redox states of the various nitrogen species are depicted at the bottom of the figure.

Anammox (abbreviation of "anaerobic ammonium oxidation") is an important microbe-mediated process in the global nitrogen cycle. The bacteria that mediate this process were discovered in 1999. This discovery solved a long-standing mystery of the global nitrogen cycle, viz., how is ammonia removed in anaerobic environments? Prior to this discovery, it was thought that oxidation of ammonia could proceed only in aerobic environments. If true, then ammonia would be expected to "pile up" in anaerobic environments; this was not observed to be the case, but the mechanism for anaerobic conversion of ammonia was unknown. This conundrum was resolved by the discovery of bacteria that could convert ammonia and nitrite directly to dinitrogen *even under anaerobic conditions*.

SOURCE: Strous M, Fuerst JA, Kramer EH, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MS (1999) Missing lithotroph identified as new planctomycete. *Nature* 400(6743): 446-449 PMID: 10440372.

Abstract: With the increased use of chemical fertilizers in agriculture, many densely populated countries face environmental problems associated with high ammonia emissions. The process of anaerobic ammonia oxidation ("anammox") is one of the most innovative technological advances in the removal of ammonia nitrogen from waste water. This new process combines ammonia and nitrite directly into dinitrogen gas. Until now, bacteria capable of anaerobically oxidizing ammonia had never been found and were known as "lithotrophs missing from nature". Here we report the discovery of this missing lithotroph and its identification as a new, autotrophic member of the order *Planctomycetales*, one of the major distinct divisions of the Bacteria. The new planctomycete grows extremely slowly, dividing only once every two weeks. At present, it cannot be cultivated by conventional microbiological techniques. The identification of this bacterium as the one responsible for anaerobic oxidation of ammonia makes an important contribution to the problem of unculturability.

Anammox (anaerobic ammonium oxidation) cycle



SOURCE: Kuenen J.G. (2008) Anammox bacteria: from discovery to application. *Nature Reviews Microbiology* 6(4): 320-326 PMID:18340342.

SOURCE: DeLong E.F. (2002) Microbiology: all in the packaging. *Nature* 419(6908): 676-677 PMID:12384680.

Figure 1. *Brocadia anammoxidans*: bacteria with a difference.

(a) Schematic of the anammox bacterium *Brocadia anammoxidans*, showing the anammoxosome. This is the membrane-bounded organelle-like structure in which the energy-generating process involving the combination of ammonia with nitrite takes place.

(b) Diagram of the anammoxosome membrane, which consists of a unique "ladderane" lipid bilayer, and the anammox reaction pathway. A key intermediate in the cycle is **hydrazine** (N_2H_4 or $H_2N{=}NH_2$), which is highly toxic and explosively reactive (during World War II it was a major component of rocket fuel!). Hence the need for containment within a special membrane-bounded compartment, the "anammoxosome". The anammoxosome's dense and impermeable ladderane membrane (see next slide) prevents N_2H_4 from leaking out of the anammoxosome into the cytoplasm.

Abbreviations: NO_2^-, nitrite; NH_2OH, hydroylamine; NH_3, ammonia; N_2H_4, hydrazine.

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Molecular mechanism of the anammox process

(1) $NO_2^- + 2H^+ + 1e^- \rightarrow NO + H_2O$
Redox couple: NO_2^-/NO (E_0' +0.38 V)

(2) $NO + NH_3 + 3H^+ + 3e^- \rightarrow N_2H_4 + H_2O$
Redox couple: $(NO+NH_3)/N_2H_4$ (E_0' +0.06 V)

(3) $N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$
Redox couple: N_2/N_2H_4 (E_0' −0.75 V)
For comparison: $NAD^+/NADH$ (E_0' −0.32 V)

Overall: $NO_2^- + NH_3 + H^+ + \rightarrow N_2 + 2H_2O$ ($\Delta G^{o\prime} = -357$ kJ * $mole^{-1}$)

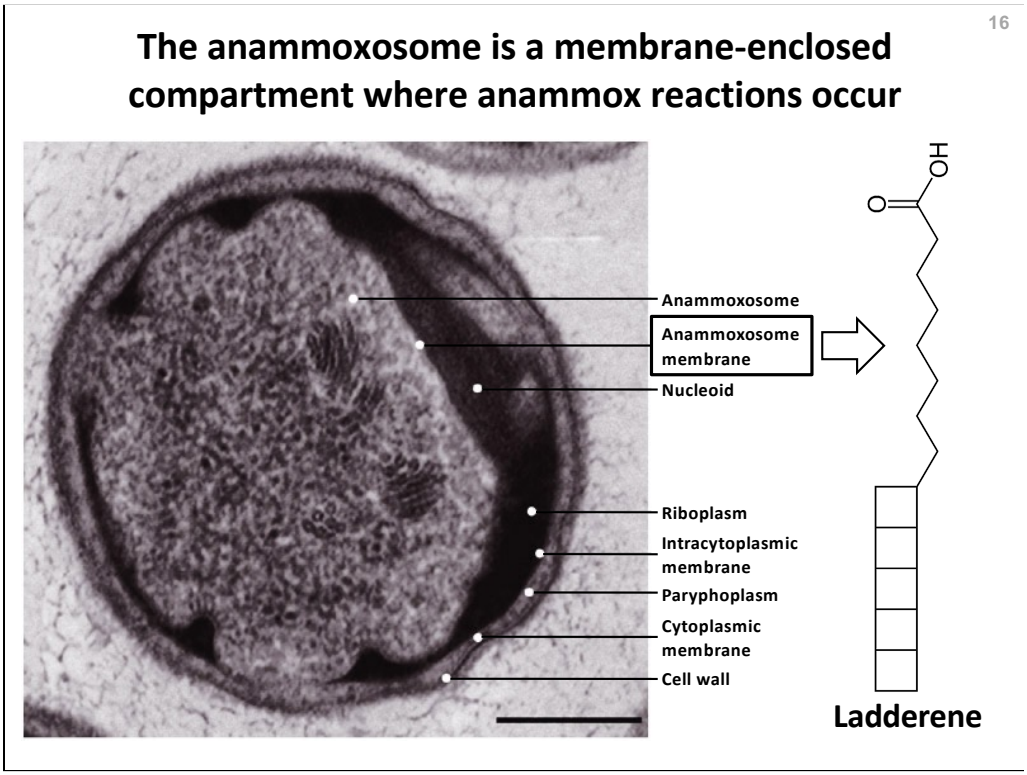
Compare: $NADH + H^+ + ½O_2 \rightarrow NAD^+ + H_2O$ ($\Delta G^{o\prime} = -228$ kJ * $mole^{-1}$)

SOURCE: Kartal B., Maalcke W.J., de Almeida N.M., Cirpus I., Gloerich J., Geerts W., Op den Camp H.J., Harhangi H.R., Janssen-Megens E.M., Francoijs K.J., Stunnenberg H.G., Keltjens J.T., Jetten M.S., Strous M. (2011) Molecular mechanism of anaerobic ammonium oxidation. *Nature* 479(7371): 127-130 PMID:21964329.

In sum: the reaction $NO_2^- + NH_3 + H^+ + \rightarrow N_2 + 2H_2O$ has a $\Delta G^{o\prime} = -357$ kJ * $mole^{-1}$.

For comparison: the familiar reaction $NADH + H^+ + ½O_2 \rightarrow NAD^+ + H_2O$ has a $\Delta G^{o\prime} = -228$ kJ * $mole^{-1}$.

Abbreviations: NO_2^-, nitrite; NO, nitric oxide; NH_2OH, hydroylamine; NH_3, ammonia; NH_4^+, ammonium ion; N_2H_4, hydrazine; N_2, dinitrogen.



SOURCE: Kuenen J.G. (2008) Anammox bacteria: from discovery to application. *Nature Reviews Microbiology* 6(4): 320-326 PMID:18340342.

Figure 2. Transmission electron micrograph of the anammox bacterium *Candidatus Kuenenia stuttgartiensis*. As a member of the phylum *Planctomycetes*, *C. Kuenenia stuttgartiensis* contains subcellular compartments that are membrane-bounded, including the enormous anammoxosome, where energy conservation takes place. The relatively small "riboplasm" is the equivalent of the ribosome-containing cytoplasm in most other bacteria. Scale bar, 200 nm.

The anammoxosome membrane is composed of unique lipids ("ladderenes") built from concatenated cyclobutane rings, which form a molecular ladder. These molecules render the anammoxosome membrane impermeable to hydrazine (N_2H_4), a highly reactive and toxic compound that is formed within the organelle as an intermediate in the anammox reaction sequence. To date, ladderenes have been found only in association with anammox bacteria, and thus can be used to identify these organisms in their natural environments.

Annamox bacteria were first identified in 1999 by Strous *et al.* (1999) Missing lithotroph identified as new planctomycete. *Nature* 400(6743): 446-449 PMID:10440372.

17

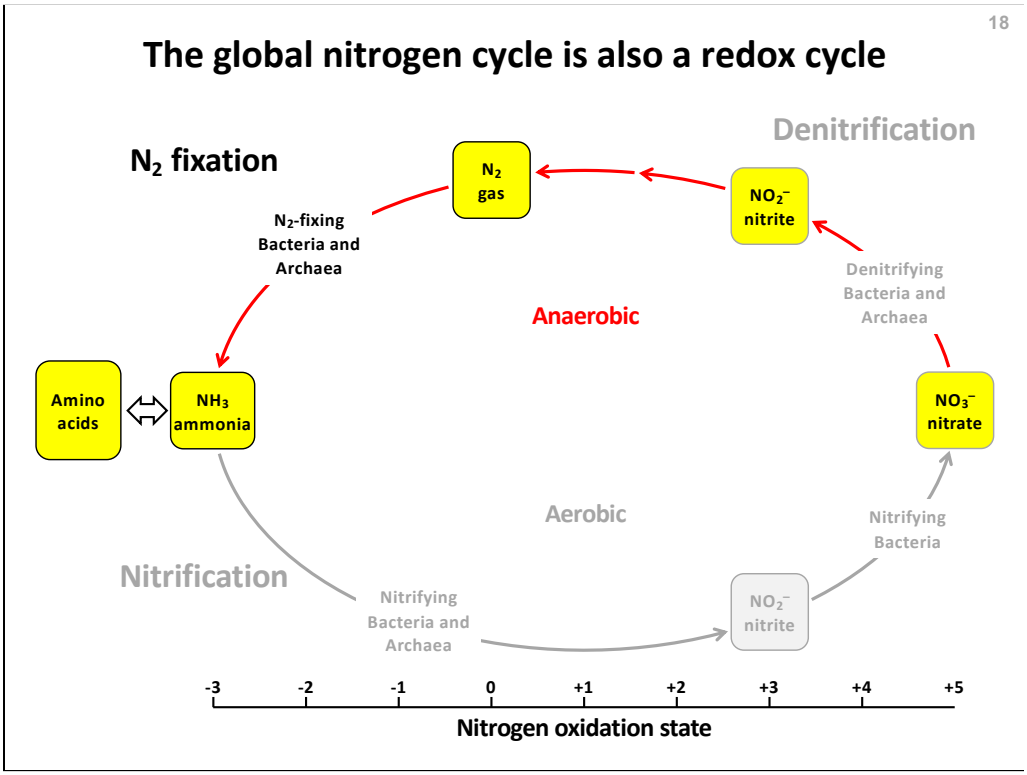
The ANAMMOX® (Anaerobic Ammonium Oxidation) process is used to remove ammonia from wastewater

SOURCE: http://paques.hscglab.nl/?pid=46

SOURCE: http://biotechind2.altervista.org/lezioni/anammox/application.html

ANAMMOX® (Anaerobic Ammonium Oxidation) is a biological process representing a major breakthrough in nitrogen waste removal. The ANAMMOX® process is a cost-effective and sustainable way of removing ammonium from waste water and ammonia from waste gas. Compared to conventional nitrification/denitrification systems the operational costs are reduced by 90%, power consumption is reduced by 60%, and CO_2 emissions are reduced by 90%. Furthermore, the ANAMMOX® plant requires less than 50% of the space taken up by conventional processes. The ANAMMOX® conversion is an elegant shortcut in the natural nitrogen cycle. In combination with nitritation, ANAMMOX® bacteria convert ammonia (NH_3) directly into dinitrogen (N_2) gas. In collaboration with the Delft University of Technology, Paques has developed this process for commercial purposes. In the summer of 2002 the first full-scale ANAMMOX® plant was started up in the Netherlands.

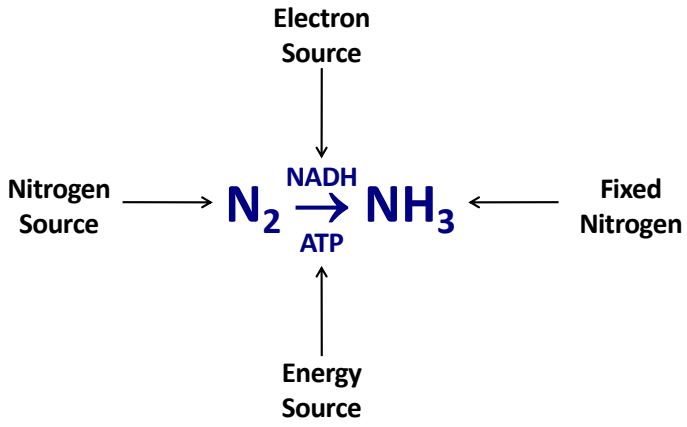
Applications: The ANAMMOX® process can be used for the removal of ammonium from all types of effluents with a relatively high ammonia concentration (NH_3 > 100 mg/liter). Processes and industries where these effluents are found include: • municipal waste water treatment (rejection water from a sludge digester) • organic solid waste treatment (landfills, composting, digestion) • food industries • manure processing industries • fertilizer industries • (petro)chemical industries • metallurgical industries • mining industries.



SOURCE: *Lehninger Principles of Biochemistry* (p. 852), published by W.H. Freeman & Co., New York, NY © 2008

Figure 22.1. The nitrogen cycle. The total amount of nitrogen fixed annually is the biosphere exceeds 100,000,000,000 (10^{11}) kg. Reactions with red arrows occur in anaerobic environments. The redox states of the various nitrogen species are depicted at the bottom of the figure.

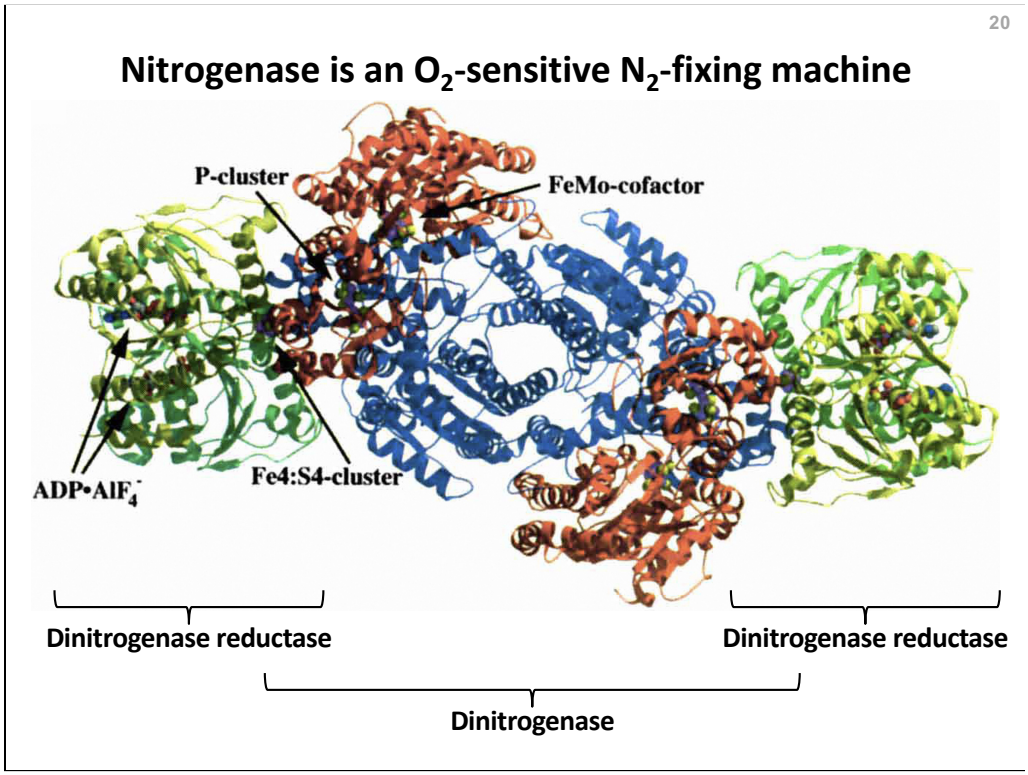
Nitrogen fixation: all life on earth depends on this deceptively simple chemical transformation



Nitrogen fixers include Archaea and Bacteria but not Eukarya!

SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019.

Chapter 14.6: Nitrogen fixation. The biological utilization of dinitrogen (N_2) as cell nitrogen is called "nitrogen fixation". The N_2 is reduced to NH_3 (ammonia), a major form of fixed nitrogen, and then assimilated into organic forms, such as amino acids and nucleotides. The ability to fix nitrogen frees an organism from dependence on fixed nitrogen in its environment and confers a significant ecological advantage on it. The process of nitrogen fixation is also of enormous agricultural importance, supporting the nitrogen needs of key crops, such as soybeans, which are able to fix nitrogen thanks to nitrogen-fixing bacterial (rhizobial) endosymbionts living in root nodules, which we will address later in this lecture

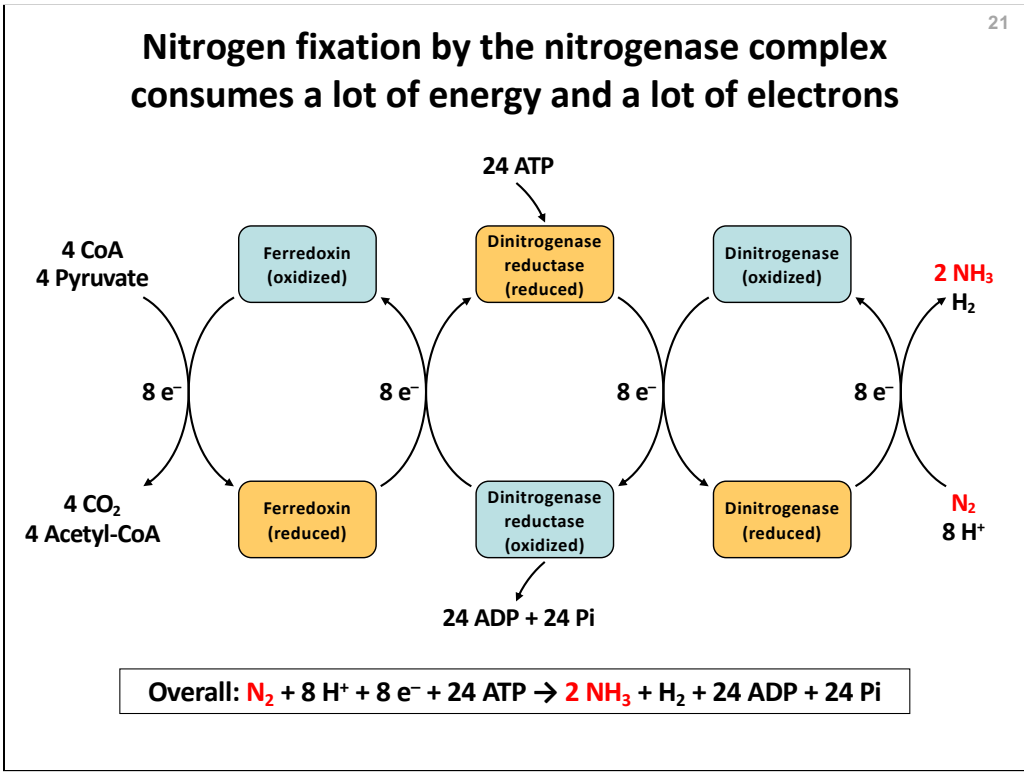


SOURCE: Schindelin H., Kisker C., Schlessman J.L., Howard J.B., Rees D.C. (1997) Structure of ADP x AIF4(-)-stabilized nitrogenase complex and its implications for signal transduction. *Nature* 387(6631): 370-376 PMID:9163420.

Figure 1. Overall structure of the nitrogenase complex from *Azotobacter vinlandyii*. The entire complex with the molybdenum-iron (MoFe) alpha-subunits in red, the beta-subunits in blue, and the individual subunits of each Fe-protein in green and yellow. The cofactors and the bound nucleotides are shown in ball-and-stick representation. Atoms are color-coded, with Al in orange, Mg and Mo in cyan, Fe and P in purple, S in yellow, O in red, N in blue, and C in grey.

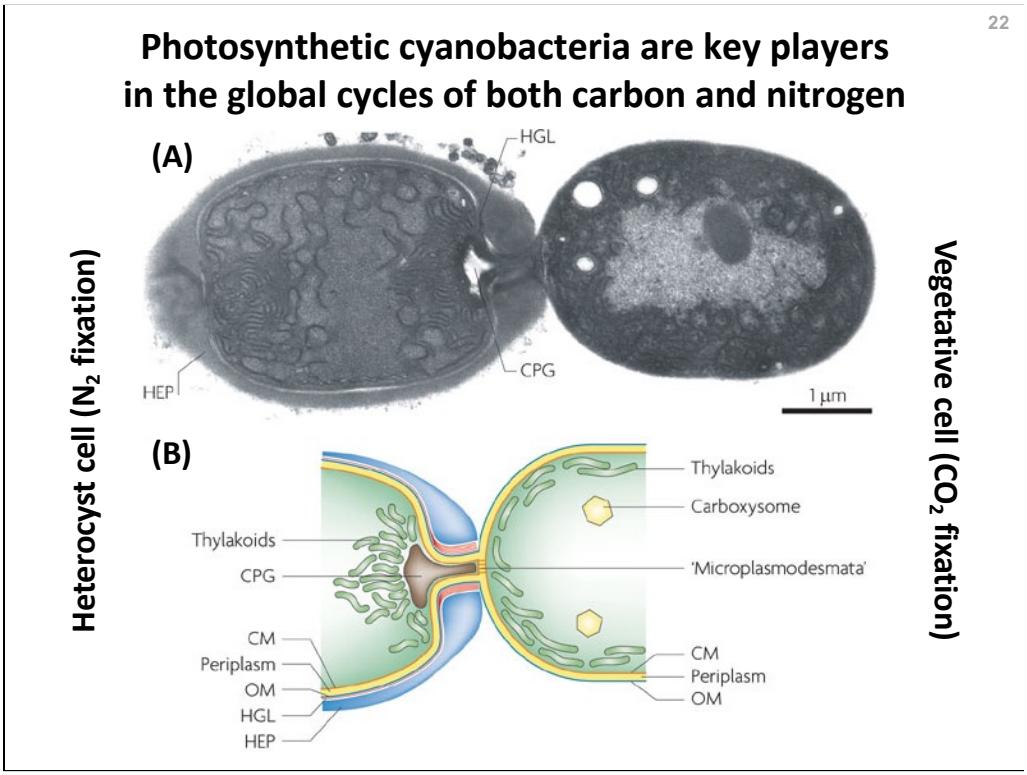
The FeMo (iron-molybdenum) cofactor probably represents the active site for substrate reduction. The P-cluster appears to serve as the intermediate electron transfer center between the Fe-protein donor and the FeMo-cofactor.

ADP•AlF_4^- is ADP stabilized by interaction with aluminum fluoride.



SOURCE: *Lehninger Principles of Biochemistry* (p. 855), published by W.H. Freeman & Co., New York, NY © 2008.

Figure 22.2. Nitrogen fixation by the nitrogenase complex. Electrons are transferred from pyruvate to dinitrogenase via ferredoxin (or flavodoxin) and dinitrogenase reductase. Dinitrogenase reductase reduces dinitrogenase one electron at a time, with at least six electrons required to fix one molecule of N_2. An additional two electrons are used to reduce 2 H^+ to H_2 in a process that obligatorily accompanies nitrogen fixation in anaerobes, making a total of eight electrons required per N_2 molecule reduced.



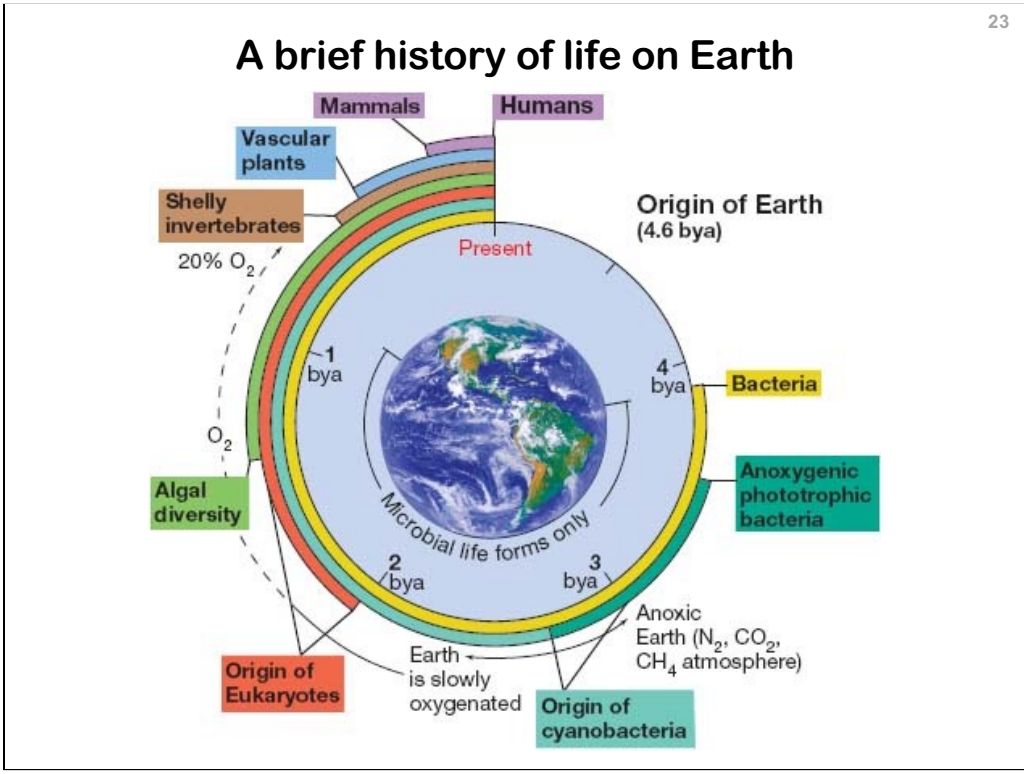
SOURCE: Flores E, Herrero A (2010) Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol* 8(1): 39-50 PMID:19966815.

Figure 4. The heterocyst and the heterocyst-vegetative cell septum.

(A) Transmission electron micrograph showing a terminal nitrogen-fixing heterocyst (left) and an adjacent carbon-fixing vegetative cell (right) from a filament of the cyonaobacterium *Anabaena* sp. PCC 7120. In preparation of samples for electron microscopy, the cyanophycin granule (CPG) is lost, leaving a white, empty space in the micrograph.

(B) Schematic of portions of a heterocyst and an adjacent vegetative cell. A continuous periplasm that is delimited by the cytoplasmic membrane (CM) and outer membrane (OM) and contains the peptidoglycan runs along the filament. Thus, all cells within a filament share the same periplasmic space. In the septum between the two cells, and in the septa between vegetative cells along the filament, thin structures called "microplasmodesmata" are present. In the heterocyst, external to the outer membrane, specific glycolipid and polysaccharide layers are present. The different distributions of thylakoids in the two types of cells (peripheral in vegetative cells and polar in the heterocyst) and the presence of carboxysomes in vegetative cells but not in heterocysts are indicated.

Abbreviations: HEP, heterocyst polysaccharide layer; HGL, heterocyst glycolipid layer.

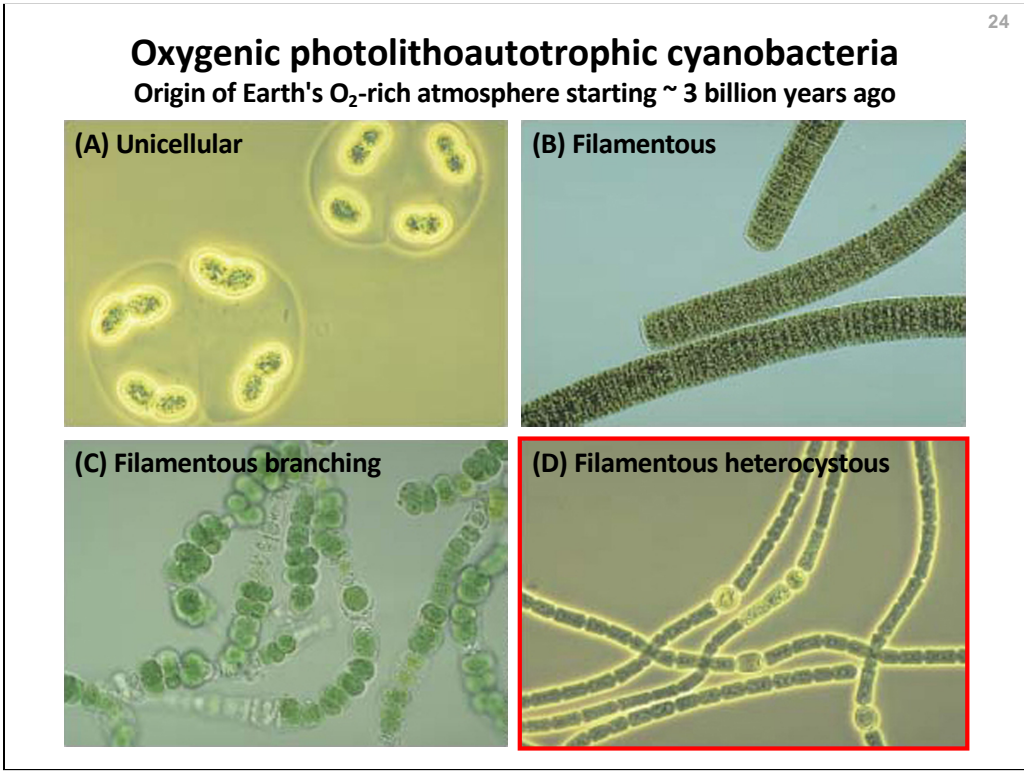


SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 1: The Microbial World (pp. 37-69), published by Pearson Education Inc., San Francisco © 2019.

Figure 1.5. A summary of life on Earth through time. Cellular life appeared on Earth about 3.8 billion years ago. Starting about 3 billion years ago, cyanobacteria began the slow oxygenation of Earth via oxygenic photosynthesis, but the current high levels of oxygen in the atmosphere were not achieved until about 500-800 million years ago. Eukaryotes appeared about 2 billion years ago; eukaryotes are nucleated cells and include both microbial and multicellular organisms

Earth is about 4.6 billion years old. There is evidence that cells first appeared on Earth between 3.8 and 3.9 billion years ago; these organisms were exclusively microbial. In fact, microorganisms were the only life on Earth for most of its history. Gradually, and over enormous periods of time, "higher" organisms appeared. What were some of the highlights along the way? During the first 2 billion years or so of Earth's existence, the atmosphere was anoxic; oxygen was absent, and nitrogen (N_2), carbon dioxide (CO_2), and a few other gases were present. Only microorganisms capable of anaerobic metabolisms could survive under these conditions, but these included many different types of cells, including those that produce methane, called methanogens. The evolution of phototrophic microorganisms - organisms that harvest energy from sunlight - occurred within a billion years after the formation of Earth. The first phototrophs were relatively simple ones, such as the purple bacteria and their relatives, which are still widespread in anoxic habitats today. Cyanobacteria evolved from these early phototrophs nearly a billion years later and began the long slow process of oxygenating the atmosphere via oxygenic photosynthesis. Facilitated by increases in oxygen in the atmosphere, multicellular life forms eventually evolved, culminating in the plants and animals we know today. How do we know that events in the evolution of life occurred as depicted in the figure? The answer is that we will probably never know for sure. However, microbiologists have identified key chemical components in present-day organisms that are unique biomarkers for particular groups. Traces of many of these biomarkers can still be found in ancient rocks. The timeline shown in the figure pieces together what we know of molecular fossils from rocks of specific ages. Over time, microorganisms diversified and came to colonize every habitat on Earth that would support life, including many habitats unsuitable for other life forms.

Not shown on this slide, but nitrogen fixation is thought to be an ancient trait that evolved sometime between 3.5 and 2.2 billion years ago. Source: Mathesius U (2022) Are legumes different? Origins and consequences of evolving nitrogen fixing symbioses. *J. Plant Physiol.* 276: 153765 PMID: 35952452.



SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 15: Functional Diversity of Microorganisms (pp. 487-529), published by Pearson Education Inc., San Francisco © 2019.

Figure 15.2. Cyanobacteria: the five major morphological types of cyanobacteria.

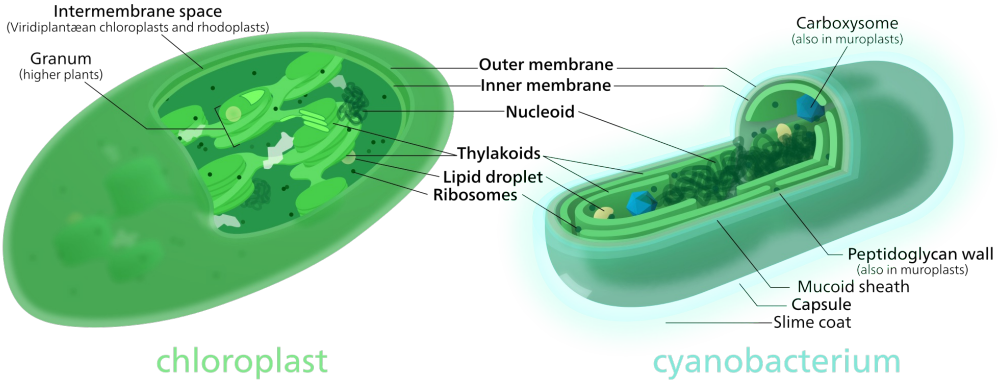
(A) Unicellular, *Gloeothece*, phase contrast; a single cell measures 5-6 µm in diameter.

(B) Filamentous, *Oscillatoria*, bright field; a single cell measures about 15 µm wide.

(C) Filamentous branching, *Fischerella*, bright field.

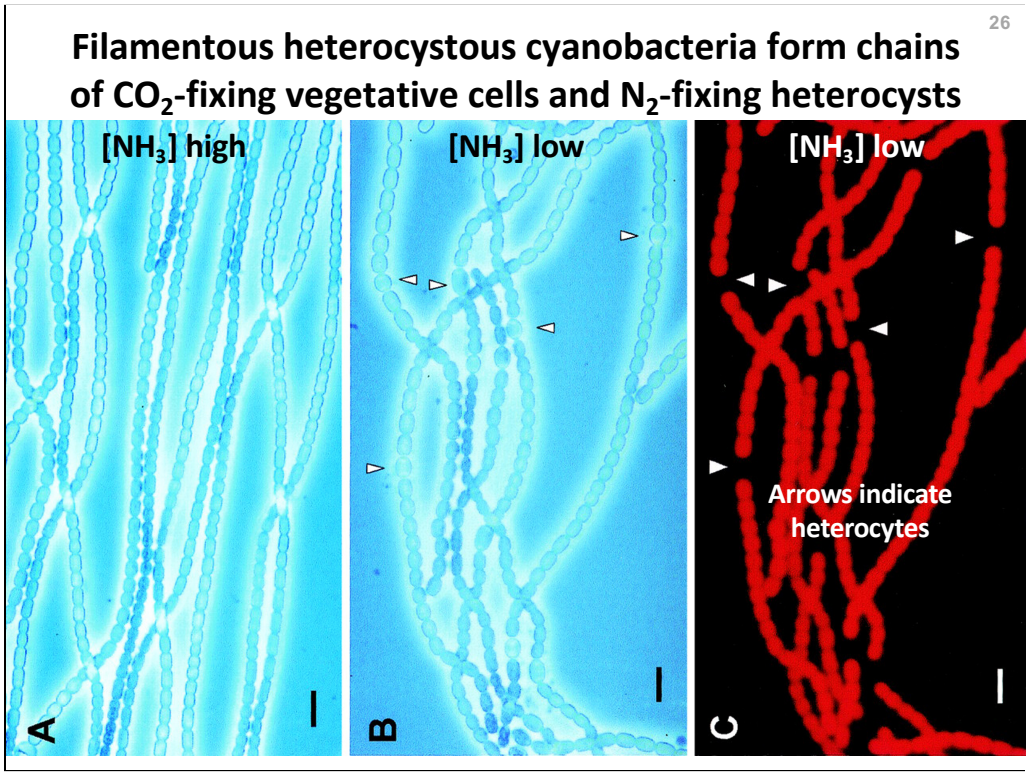
(D) Filamentous heterocystous, *Anabaena*, phase contrast; a single cell measures about 5 µm wide.

Oxygenic photolithoautotrophic cyanobacteria evolved into endosymbiotic chloroplasts in plants



SOURCE: http://en.wikipedia.org/wiki/Cyanobacteria.

Cyanobacteria (Gram-negative) are found in almost every conceivable environment, from oceans to fresh water to bare rock to soil. They can occur as planktonic cells or form phototrophic biofilms in fresh water and marine environments, they occur in damp soil, or even temporarily moistened rocks in deserts. A few are endosymbionts in lichens, plants, various protists, or sponges and provide energy for the host. Cyanobacteria include unicellular and colonial species. Colonies may form filaments, sheets, or even hollow balls. Some filamentous colonies show the ability to differentiate into several different cell types: **vegetative cells**, the normal, photosynthetic cells that are formed under favorable growing conditions; **akinetes**, the climate-resistant spores that may form when environmental conditions become harsh; **hormogonia**, motile filaments that travel away from the main biomass to bud and form new colonies elsewhere; and thick-walled **heterocysts**, which contain the enzyme **nitrogenase**, vital for nitrogen fixation. Heterocysts may also form under the appropriate environmental conditions (anoxic) wherever nitrogen is necessary. Heterocyst-forming species are specialized for nitrogen fixation and are able to fix nitrogen gas (N_2), which cannot be used by plants, into ammonia (NH_3), nitrites (NO_2^-), or nitrates (NO_3^-), which can be absorbed by plants and converted to protein and nucleic acids. The rice paddies of Asia, which produce about 75% of the world's rice, could not do so were it not for nitrogen-fixing cyanobacteria in the rice paddy fertilizer. Some species of cyanobacteria contribute significantly to global ecology and the oxygen cycle. The tiny marine cyanobacterium *Prochlorococcus* (discovered in 1986) accounts for more than half of the photosynthesis in the open ocean.



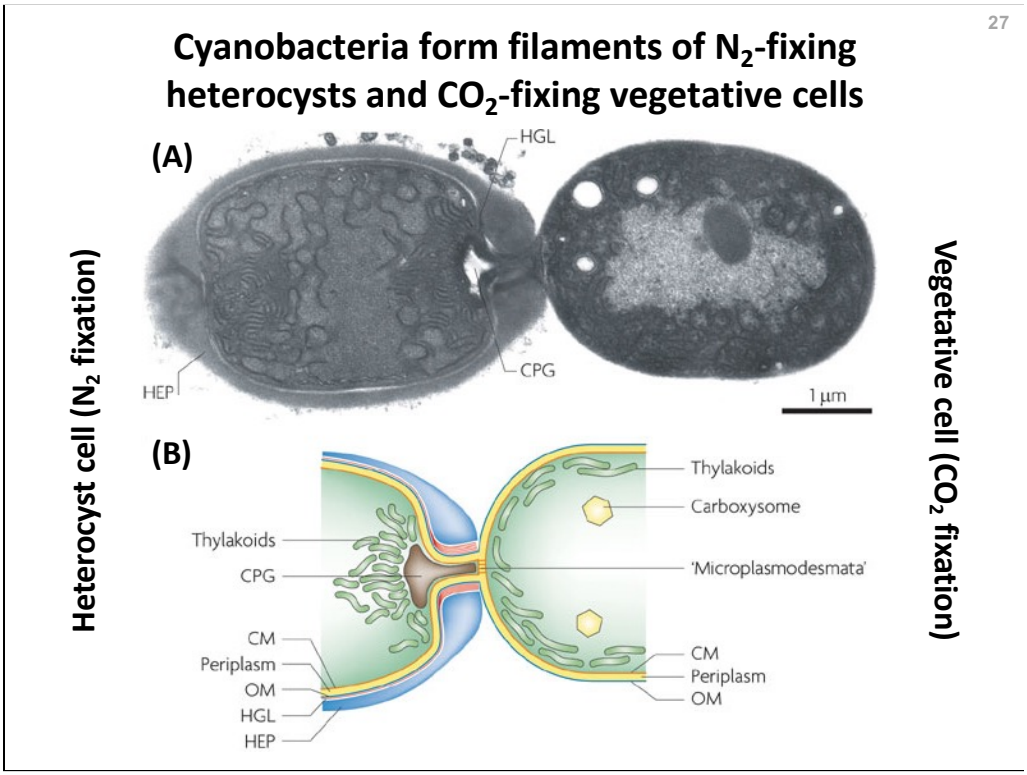
SOURCE: Meeks JC, Elhai J (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol. Mol. Biol. Rev.* 66(1): 94-121 PMID:11875129.

Figure 1. Photomicrographs of vegetative and heterocyst-containing filaments of the cyanobacterium *Nostoc punctiforme*.

(A) Phase-contrast image of vegetative filaments grown in the presence of ammonia (NH_3). No heterocysts are visible.

(B) Phase-contrast image of filaments grown in the absence of any fixed nitrogen source in the culture medium. Heterocysts, identified by arrowheads, are present at evenly-spaced intervals.

(C) Epifluorescence image of the same filaments as in panel B. Heterocysts have negligible fluorescence, while vegetative cells have intense combined fluorescence from phycobiliproteins and chlorophyll *a* used in photosynthesis. Scale bar, 10 µm.



SOURCE: Flores E, Herrero A (2010) Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol* 8(1): 39-50 PMID:19966815.

Figure 4. The heterocyst and the heterocyst-vegetative cell septum.

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(B) Schematic of portions of a heterocyst and an adjacent vegetative cell. A continuous periplasm that is delimited by the cytoplasmic membrane (CM) and outer membrane (OM) and contains the peptidoglycan runs along the filament. Thus, all cells within a filament share the same periplasmic space. In the septum between the two cells, and in the septa between vegetative cells along the filament, thin structures called "microplasmodesmata" are present. In the heterocyst, external to the outer membrane, specific glycolipid and polysaccharide layers are present. The different distributions of thylakoids in the two types of cells (peripheral in vegetative cells and polar in the heterocyst) and the presence of carboxysomes in vegetative cells but not in heterocysts are indicated.

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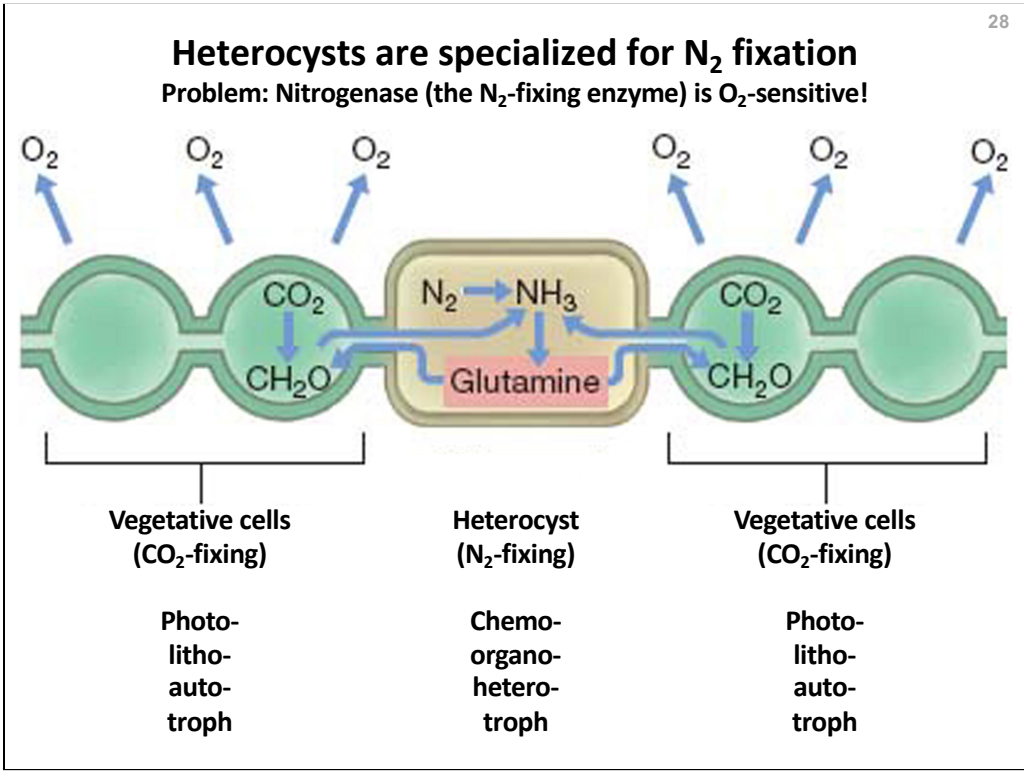
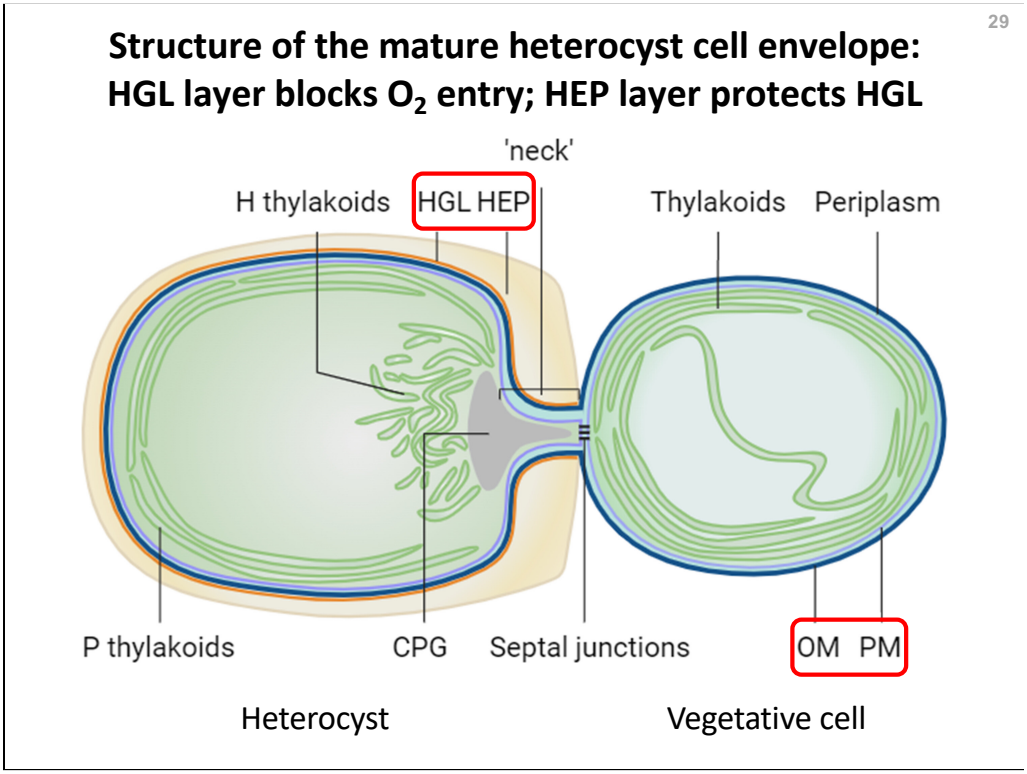


SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 7: Molecular Biology of Microbial Growth (pp. 238-258), published by Pearson Education Inc., San Francisco © 2019. Figure 7.17.

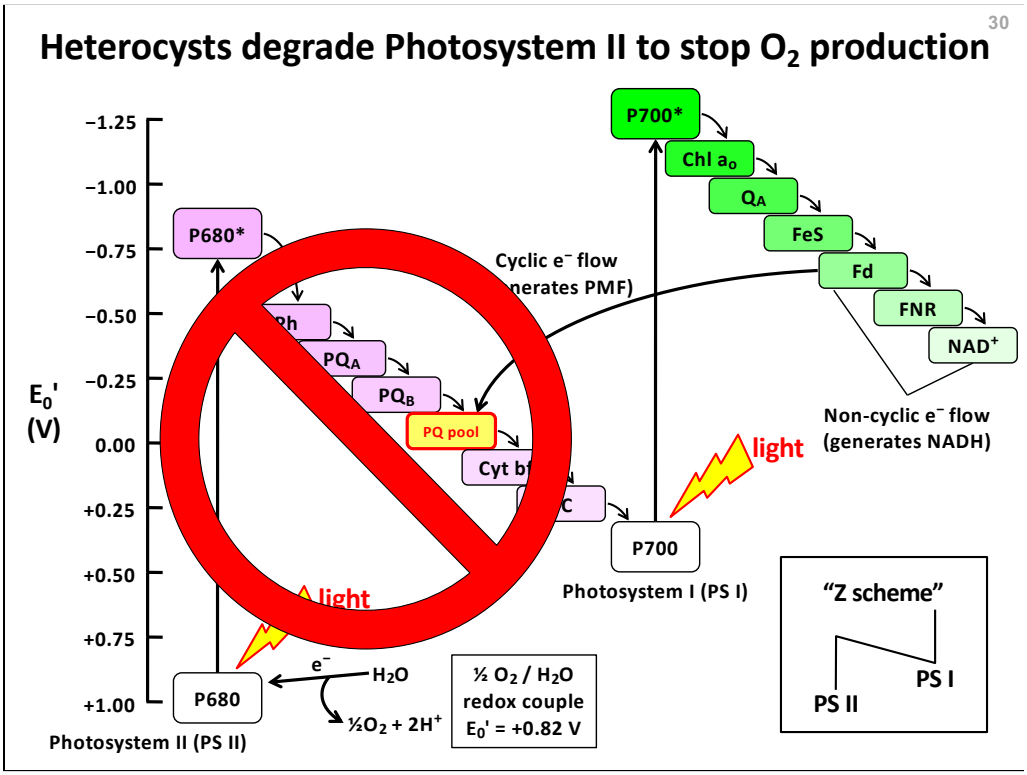
Model for the metabolic cooperation between vegetative cells and heterocysts in a filament of cyanobacteria. Vegetative cells are specialized to perform carbon fixation (CO_2-fixation) via oxidative phosphorylation, which generates molecular oxygen (O_2). This is a problem for nitrogen fixation (N_2-fixation) because O_2 rapidly and irreversibly inactivates the key enzyme (nitrogenase) that catalyzes N_2-fixation. Heterocyts are specialized to perform N_2-fixation. They shut down CO_2-fixation via oxygenic photosynthesis to prevent internal O_2 production. They also modify the cell envelope to make it impermeable to environmental O_2 (see next slide).

The **vegetative cell is a photo-litho-auto-troph** because it gets its energy from light, electrons from water, and carbon from CO_2. The vegetative cell "cross-feeds" a portion of the fixed carbon compounds that it makes to the heterocyst, which is unable to perform CO_2-fixation, through selective cell-cell junctions. The **heterocyst is a chemo-organo-hetero-troph** because it gets its energy, electrons, and carbon from the fixed carbon compounds that it receives from the vegetative cell. In return, the heterocyst "cross-feeds" a portion of the fixed nitrogen compounds that it makes to the heterocyst, which is unable to perform N_2-fixation, through selective cell-cell junctions. Glutamine is the form of fixed nitrogen transported from heterocysts to vegetative cells.



Source: Wang K, Mahbub M, Mastroianni G, Valladares A, Mullineaux CW (2024) mRNA localization and thylakoid protein biogenesis in the filamentous heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120. *J Bacteriol* 206(10):e0032824 PMID: 39329528.

Figure 1. Cartoon to illustrate the layout of thylakoid membranes in *Anabaena*. The cell on the left is a heterocyst, and the cell on the right is a vegetative cell. During heterocyst differentiation, thylakoid membranes (shown in green) are remodeled to form two distinct domains: P thylakoids (peripheral thylakoids) and H thylakoids (honeycomb thylakoids). The heterocyst-specific Cox2 and Cox3 terminal oxidases are located in the H thylakoids in mature heterocysts. CPG, cyanophycin granule; HGL, heterocyst specific glycolipid; HEP, heterocyst envelope polysaccharide; OM, outer membrane; PM, plasma membrane.



Under conditions of fixed-nitrogen starvation, a fraction of carbon-fixing vegetative cells within a cyanobacteria filament differentiate into nitrogen-fixing heterocysts. The key enzyme for nitrogen fixation (nitrogenase) is rapidly and irreversibly inactivated by O_2. This is a problem because O_2 is generated internally during carbon fixation by oxygenic photosynthesis. Heterocysts solve this problem by degrading Photosystem II, which is responsible for generating O_2 when it splits water in order to "steal" water's electrons.

Degradation of Photosystem II solves the oxygen problem, but by disrupting photosynthesis is creates another problem: how can the heterocyst obtain the organic fixed-carbon compounds that it needs to survive? The heterocyst solves this problem by obtaining fixed-carbon compounds (in the form of sugars) from the adjacent carbon-fixing vegetative cells, as illustrated on the next slide. It then uses these sugars as a source of energy, electrons, and carbon; in other words, the heterocyst is a chemo-organo-hetero-troph.

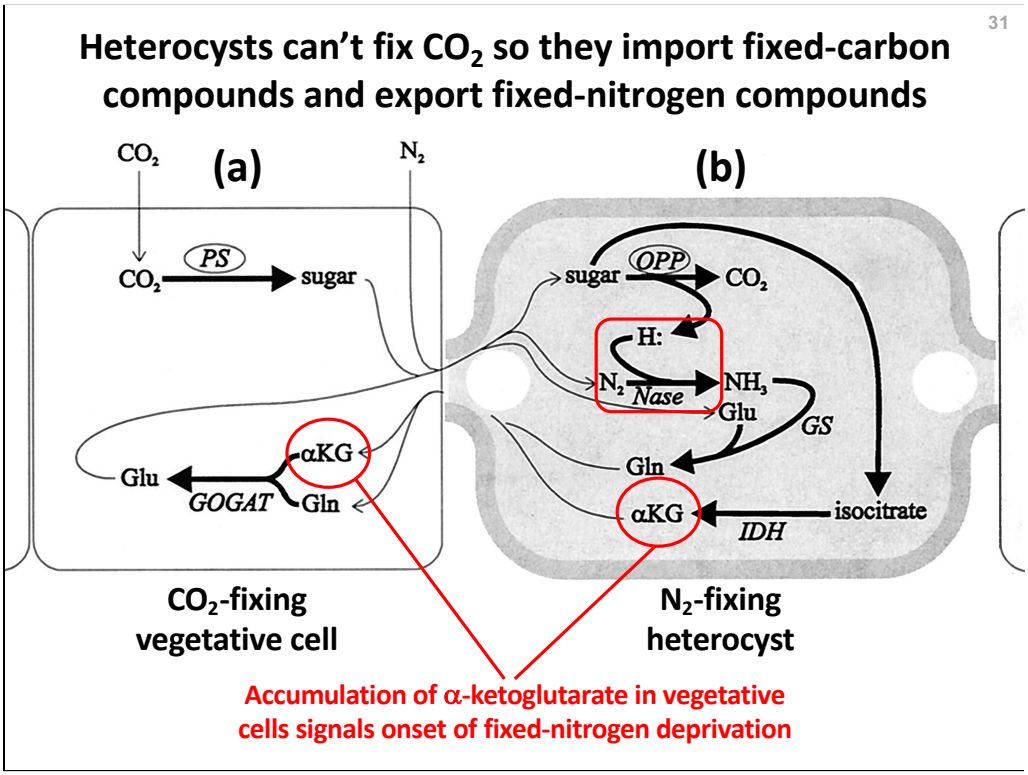
SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 14: Metabolic Diversity of Microorganisms (pp. 428-486), published by Pearson Education Inc., San Francisco © 2019. Figure 14.14.

Electron flow in oxygenic photosynthesis: the 'Z scheme'. Electrons flow through two photosystems, PS I and PS II. Compare with anoxygenic photosynthesis (Figure 14.12).

Abbreviations: **PSII**, photosystem II; **P680**, reaction center chlorophyll of PSII; **Ph**, pheophytin; **PQ**, plastoquinones (A and B); **Cyt *bf***, cytochrome *bf*; **PC**, plastocyanin; **PSI**, photosystem I; **P700**, reaction center chlorophyll of PSI; **Chl a_o**, chlorophyll a_o, **Q_{Aoxi}**, quinone A oxidized form; **Q_{Ared}**, quinone A reduced form; **FeS**, non-heme iron-sulfur protein; **Fd**, ferredoxin; **FNR**, ferredoxin NADP reductase.

Electron donor: H_2O, water.

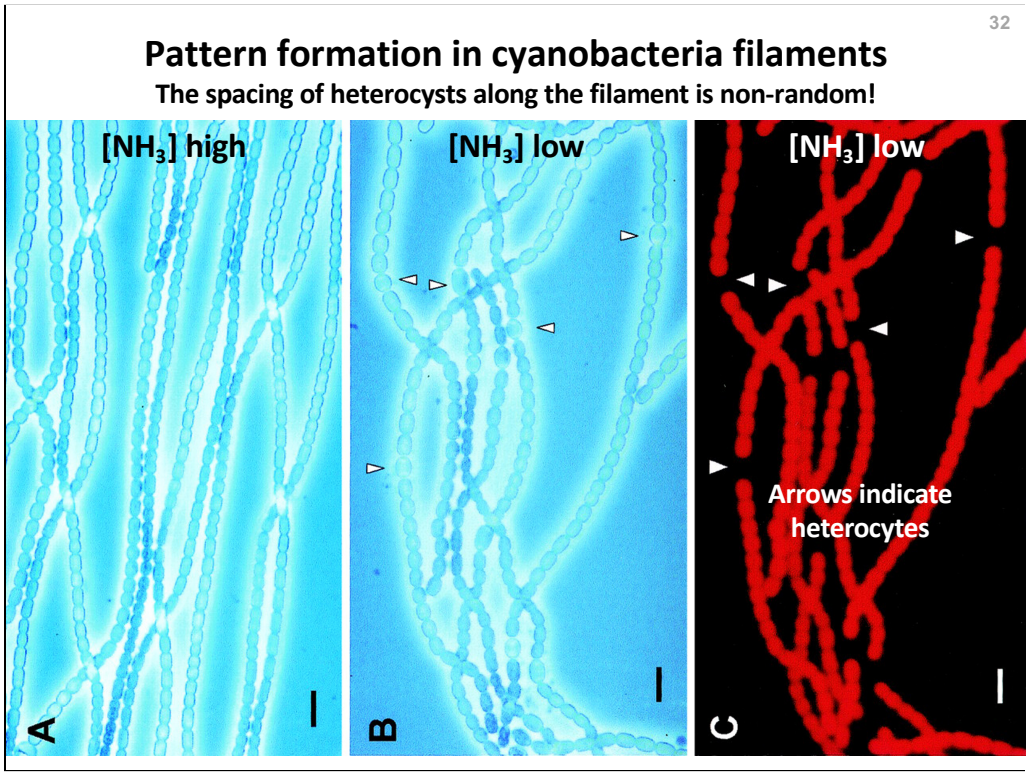
Remember: $\Delta G_0' = -n * F * \Delta E_0'$ where n = the number of electrons transferred and F (Faraday's Constant) = 96.5 kJ * V^{-1} * $mole^{-1}$ (although 100 kJ * V^{-1} * $mole^{-1}$ is precise enough for our purposes in this course.)



SOURCE: Meeks JC, Elhai J (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol Mol Biol Rev* 66(1): 94-121 PMID:11875129. Figure 3.

Metabolic interactions between vegetative cells (left) and heterocysts (right). A vegetative cell exchanges metabolites (thin lines) with a heterocyst bounded by its characteristic thick and gas-impermeable cell envelope. The heterocyst has polar plugs at either end that function as highly selective permeability barriers between the heterocyst and the adjacent vegetative cells. Thick lines indicate metabolic pathways. Carbon dioxide (CO_2) is fixed in vegetative cells through the dark reactions of photosynthesis (PS), and the resulting triose is metabolized to pyruvate through the partial citric acid cycle to isocitrate and then via isocitrate dehydrogenase (IDH) to alpha-ketoglutarate (αKG). Alpha-ketoglutarate combines with glutamine (Gln) via glutamate synthase (GOGAT) to form two molecules of glutamate (Glu). In heterocysts, sugars obtained from vegetative cells enter the oxidative pentose phosphate (OPP) pathway to produce reductant (H:) in the form of pyruvate, which is used for nitrogen fixation by nitrogenase (Nase) to produce ammonia (NH_3) and concurrently yield alpha-ketoglutarate (αKG). NH_3 combines with glutamate, obtained from the vegetative cell, through a reaction catalyzed by glutamine synthetase (GS) to form glutamine (Gln). Thus, with each turn of this cycle, one N_2 molecule is converted to two NH_3 molecules, and some of this fixed nitrogen is fed back to the connected vegetative cells as glutamine (Gln). Glutamine can then be used as a nitrogen donor in many different cellular reactions for production of other amino acids for protein synthesis, nucleotides for DNA and RNA synthesis, etc.

See Slide 21 for further details on the reaction mechanism of the N_2-to-NH_3 conversion by nitrogenase.



SOURCE: Meeks JC, Elhai J (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol. Mol. Biol. Rev.* 66(1): 94-121 PMID:11875129.

Figure 1. Photomicrographs of vegetative and heterocyst-containing filaments of the cyanobacterium *Nostoc punctiforme*.
(A) Phase-contrast image of vegetative filaments grown in the presence of ammonia (NH_3). No heterocysts are visible.
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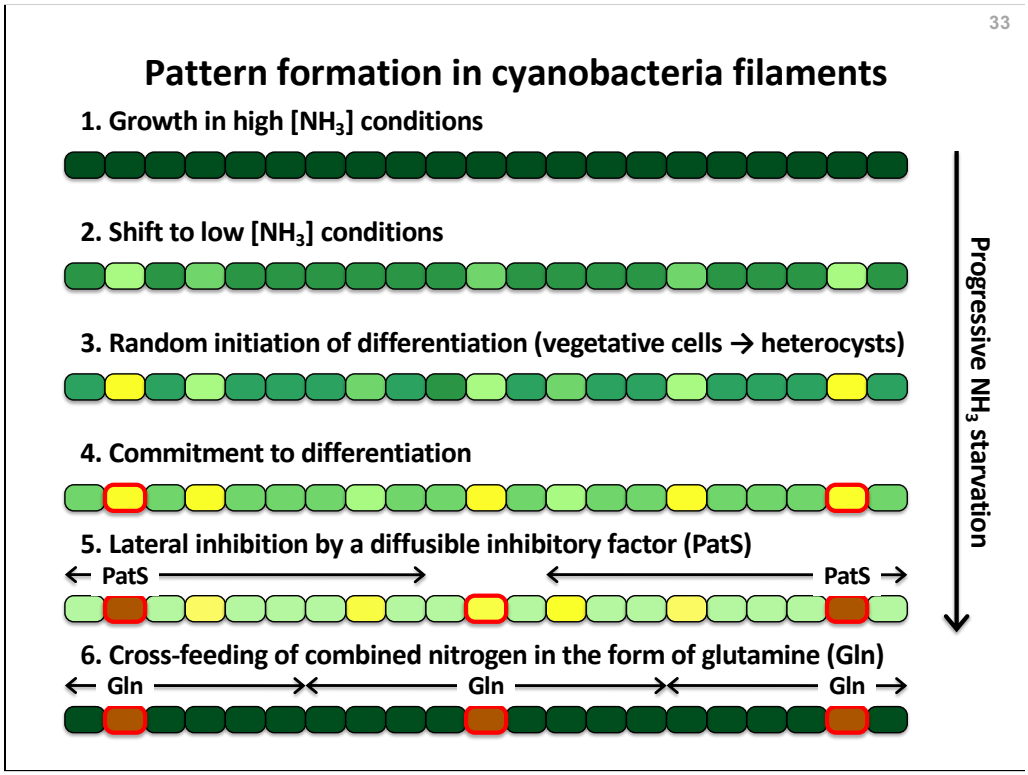
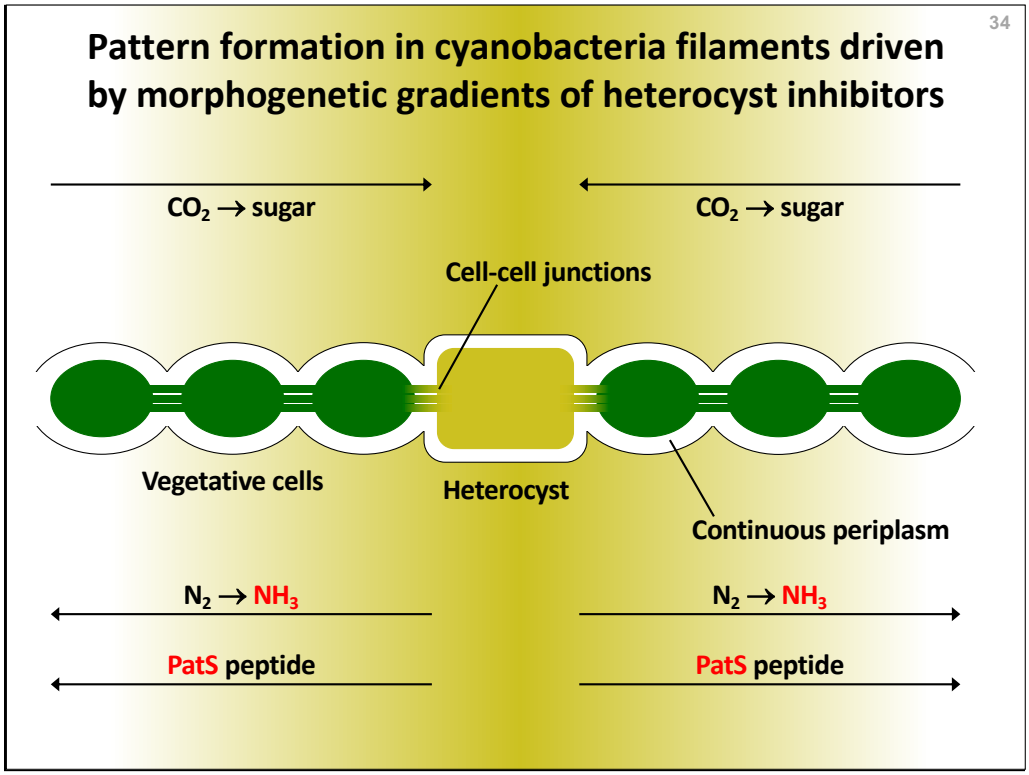
(C) Epifluorescence image of the same filaments as in panel B. Heterocysts are non-fluorescent, while vegetative cells have intense fluorescence from phycobiliproteins and chlorophyll *a* used in photosynthesis. Scale bar, 10 µm.



SOURCE: Meeks J.C., Elhai, J. (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol. Mol. Biol. Rev.* 66(1): 94-121 PMID:11875129. Figure 6.

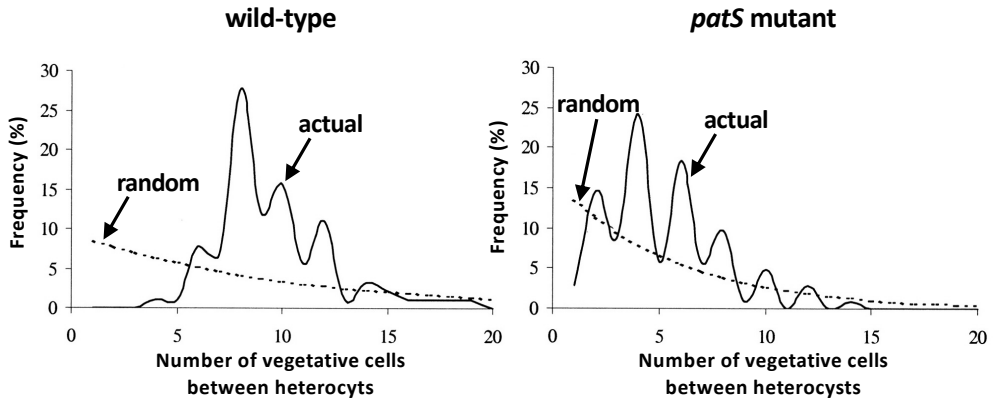
Physical interpretation of the one-stage model of heterocyst spacing. Each line represents a filament consisting of many cyanobacteria cells. The color within each cell represents its fixed-nitrogen status: the darker the color, the greater the amount of fixed nitrogen. A filament is suddenly starved for fixed nitrogen. Each cell draws on nitrogen reserves, postulated to be available in different cells to different degrees. When a cell has depleted its reserves to the extent that a critical level of starvation is reached, it becomes committed to heterocyst differentiation. Commitment has several effects. The committed cell releases a signaling peptide (**PatS**) that diffuses to adjacent cells and prevents them from differentiating. In addition, commitment prevents the committed cell from responding to its own inhibitor. Once the committed cell differentiates into a functional heterocyst capable of fixing nitrogen, it releases a second diffusible signal - a fixed-nitrogen compound such as glutamine (**Gln**) - that feeds adjacent vegetative cells (symbolized by a darkening of their color) and prevents them from differentiating into heterocysts. Cells distant from the committed cells continue to starve, until one reaches the critical level. The position of the first cells that initiate differentiation is not critical to spacing. Spacing is determined by lateral inhibition mediated by two diffusible signals originating from the heterocyst: signaling peptide (**PatS**) and a fixed-nitrogen source (**Gln**).



SOURCE: Flores E., Herrero A. (2010) Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nature Rev Microbiol* 8(1): 39-50 PMID:19966815.

Pattern formation in the cyanobacteria filament

The spacing of heterocysts along the filament is non-random



SOURCE: Meeks J.C., Elhai J. (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol. Mol. Biol. Rev.* 66(1): 94-121 PMID:11875129. Figure 5.

Spacing of heterocysts along the filament of cyanobacteria cells is non-random. Distances between heterocysts, measured as the number of vegetative cells between heterocysts, are given, and their relative frequencies by actual count (solid line) and by calculation presuming no influence of one cell on another (dashed line). *P* is defined as the probable heterocyst frequency. The value of ***α***, the probability that a cell differentiates, was taken to be the heterocyst density (number of heterocysts per total number of cells).

(A) *Anabaena* wild-type strain grown on nitrate (NO_3^-) and shifted to no fixed nitrogen for 24 hours. *P* = 10%, that is, the overall frequency is 10 heterocysts per 100 total cells.

(B) *Anabaena patS* gene-deletion mutant strain grown on nitrate (NO_3^-) and shifted to no fixed nitrogen for 24 hours. *P* = 16%, that is, the overall frequency is 16 heterocysts per 100 total cells.

Imagine that your house has a constant probability of being struck by lightning ("la foudre") at any time throughout the year. Suppose that every day the probability of a strike is the same and the rate is one strike per 100 days. Today your house was struck by lightning. What is the most likely day when lightning will strike your house **NEXT**?

A. Tomorrow.

B. After 99 days.

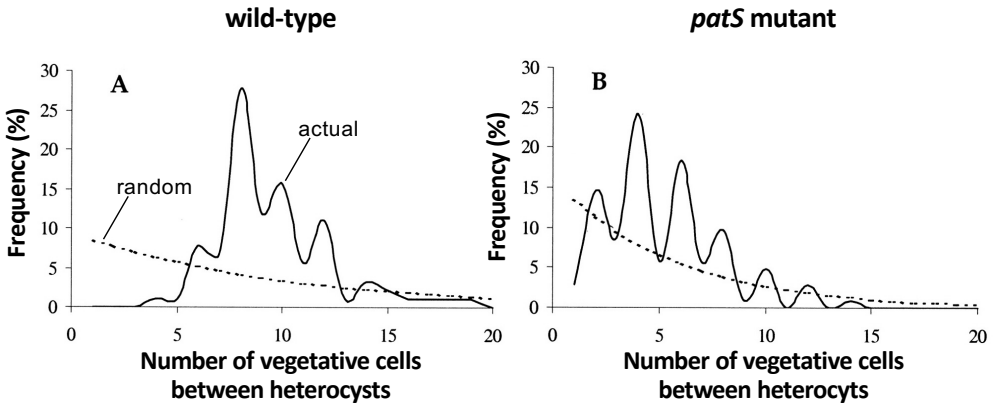
C. After 100 days.

D. Every day is equally likely to be the next day that lightning strikes.

Answer: (A)

Pattern formation in cyanobacteria filaments

The spacing of heterocysts along the filament is non-random



Random distribution if heterocyst frequency = 1 in 10 cells:

H	V1	V2	V3	V4	V5	V6	V7	V8	V9
	P = 0.1	P = 0.09	P = 0.081	P = 0.0729	P = 0.0656	P = 0.0590	P = 0.0531	P = 0.0478	P = 0.0430

SOURCE: Meeks JC, Elhai J (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol Mol Biol Rev* 66: 94-121 PMID:11875129.

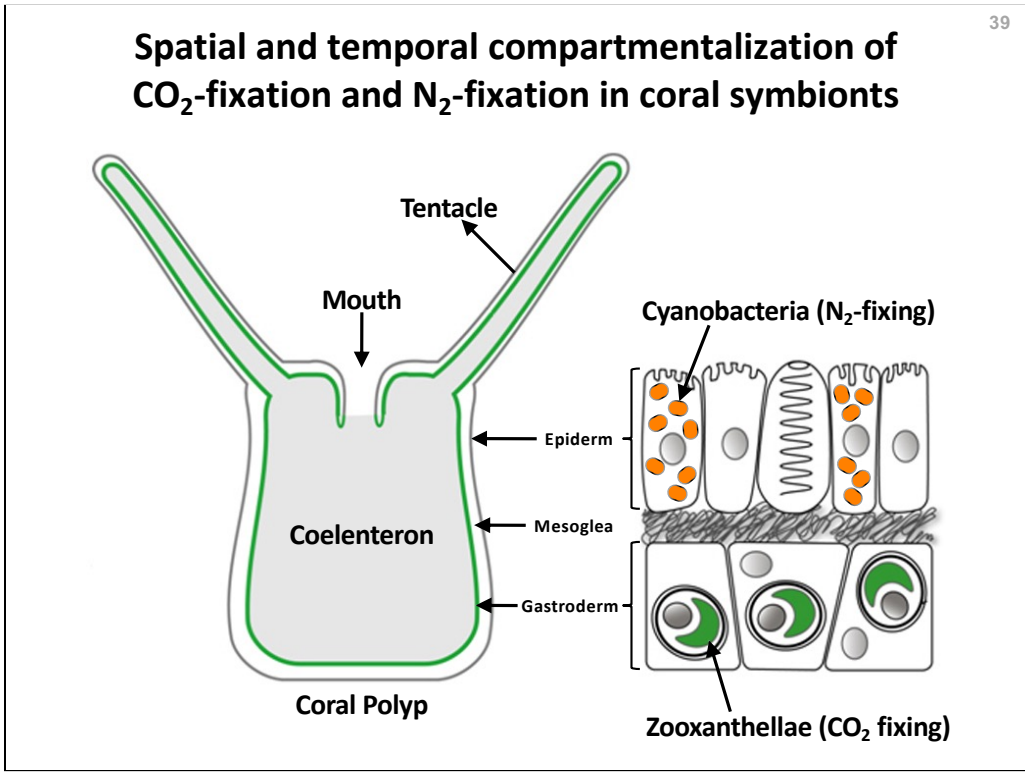
Random probability distribution of heterocyst spacing along a linear chain. This paragraph refers to the "random" probability distributions shown as dotted lines in panels (A) and (B) on the previous slide. Imagine a filament of cyanobacteria cells in which the first cell is a heterocyst (brown cell "H"), as depicted in this slide. Imagine that the overall frequency of heterocysts in such filaments is 1 in 10 ($P = 0.1$), which is about right for some species of cyanobacteria. What is the probability that the **next** vegetative cell in the chain (green cell "V1") will become a heterocyst if the switch is random? The answer of course is 0.1. OK, that part was easy. Now, moving on from cell "V1", what is the probability that the next vegetative cell in the chain (green cell "V2") will switch? The answer, of course, is 0.1, the same as for cell "V1". OK, that part was also easy. Now, here's the tricky part: what is the probability that cell "V2" will be the **next** (I emphasize the word "**next**") heterocyst in the chain after cell "H"? If you said "0.1" then you didn't pay sufficient attention to the implications of the word "**next**"! In fact, for cell "V2" to be the **next** heterocyst in the chain after cell "H", two things must happen: first, cell "V2" must switch ($P = 0.1$), and second, cell "V1" must **not** switch ($P = 0.9$). Thus, the probability that cell "V2" will become the **next** heterocyst in the chain after cell "H" is the product of the two probabilities: 0.1 x 0.9 = 0.09. Likewise, for cell "V3" to be the **next** heterocyst in the chain, three things must happen: first, cell "V3" must switch ($P = 0.1$); second, cell "V1" must not switch ($P = 0.9$); third, cell "V2" must not switch ($P = 0.9$). Thus, the probability that cell "3" will be the **next** heterocyst in the chain after cell "H" is: 0.1 x 0.9 x 0.9 = 0.081. And so on... Of course, the same thing is true moving along the filament in the opposite direction from cell "H" (moving leftwards from "H"), although this is not depicted here.

Coral polyps harbor symbionts for CO_2-fixation (zooxanthellae) and N_2-fixation (cyanobacteria)

SOURCE: Fiore C.L., Jarett J.K., Olson N.D., Lesser M.P. (2010) Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends Microbiol.* 18(10): 455-463 PMID:20674366.

Corals are marine animals in class Anthozoa of phylum Cnidaria typically living in compact colonies of many identical individual "polyps". The group includes the important reef builders that inhabit tropical oceans and secrete calcium carbonate to form a hard skeleton. A coral "head" is a colony of myriad genetically identical polyps. Each polyp is a spineless animal typically only a few millimeters in diameter and a few centimeters in length. A set of tentacles surround a central mouth opening. An exoskeleton is excreted near the base. Over many generations, the colony thus creates a large skeleton that is characteristic of the species. Individual heads grow by asexual reproduction of polyps. Corals also breed sexually by spawning. Although corals can catch small fish and plankton, using stinging cells on their tentacles, most corals obtain the majority of their energy and nutrients from photosynthetic unicellular algae called zooxanthellae that live within the coral's tissue.

In coral reef ecosystems, the major source of new nitrogen is nitrogen fixation and several important members of the reef community have the capability to fix nitrogen through mutualistic symbiotic associations with nitrogen-fixing cyanobacteria and heterotrophic bacteria. In addition to their symbiotic zooxanthellae, corals harbor a variety of bacteria associated within their tissues and endoskeleton, and although these associations are widely distributed, stable, and non-pathogenic, their function remains largely unknown. Among the diverse community of coral bacterial symbionts, many nitrogen-fixing bacteria associated with reef-building corals have been reported. Nitrogen fixation has previously been attributed to bacteria associated with living coral tissue and recent metagenomic studies have shown that many corals contain cyanobacterial genes and also the genes associated with nitrogen fixation.



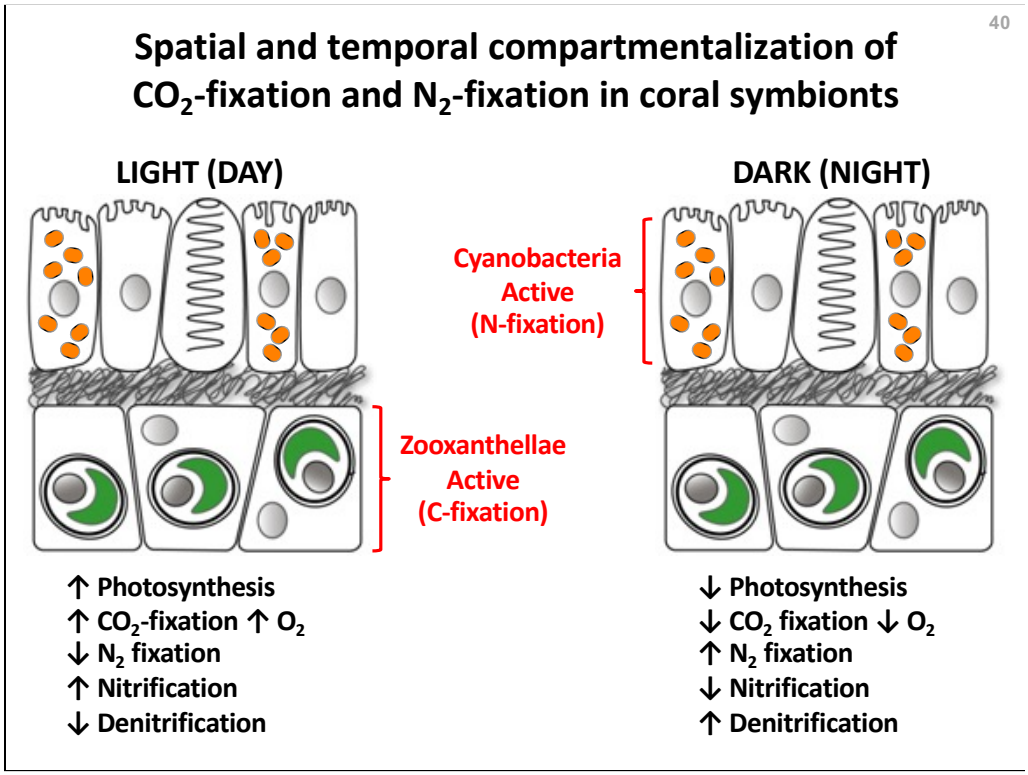
SOURCE: Sohm J.A., Webb E.A., Capone D.G. (2011) Emerging patterns of marine nitrogen fixation. *Nat Rev Microbiol* 9(7): 499-508 PMID:21677685.

SOURCE: Fiore C.L., Jarett J.K., Olson N.D., Lesser M.P. (2010) Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends Microbiol.* 18(10): 455-463 PMID:20674366. Figure 3.

A coral with typical modular tissue construction comprising repetitive and genetically identical polyps. These animals contain symbiotic carbon-fixing eukaryotic (zooxanthellae) in their gastroderm cells and symbiotic nitrogen-fixing cyanobacteria associated with the epiderm.

The **carbon-fixing zooxanthellae** are **photo-litho-auto-trophs** because they obtain their energy from light, electrons from an inorganic molecule (H_2O), and carbon from an inorganic molecule (CO_2).

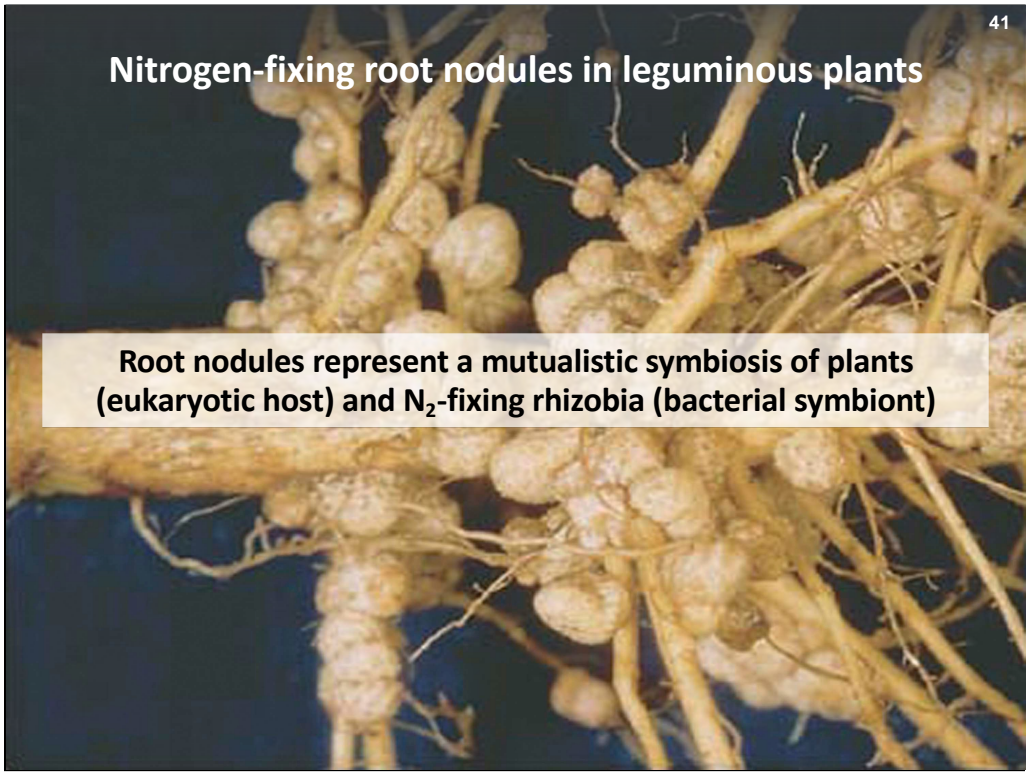
The **nitrogen-fixing cyanobacteria** are **chemo-organo-hetero-trophs** because they obtain their energy, electrons, and carbon from fixed-carbon sources, which are produced by the zooxanthellae via oxygenic photosynthesis.



SOURCE: Sohm J.A., Webb E.A., Capone D.G. (2011) Emerging patterns of marine nitrogen fixation. *Nat. Rev. Microbiol.* 9(7): 499-508 PMID:21677685.

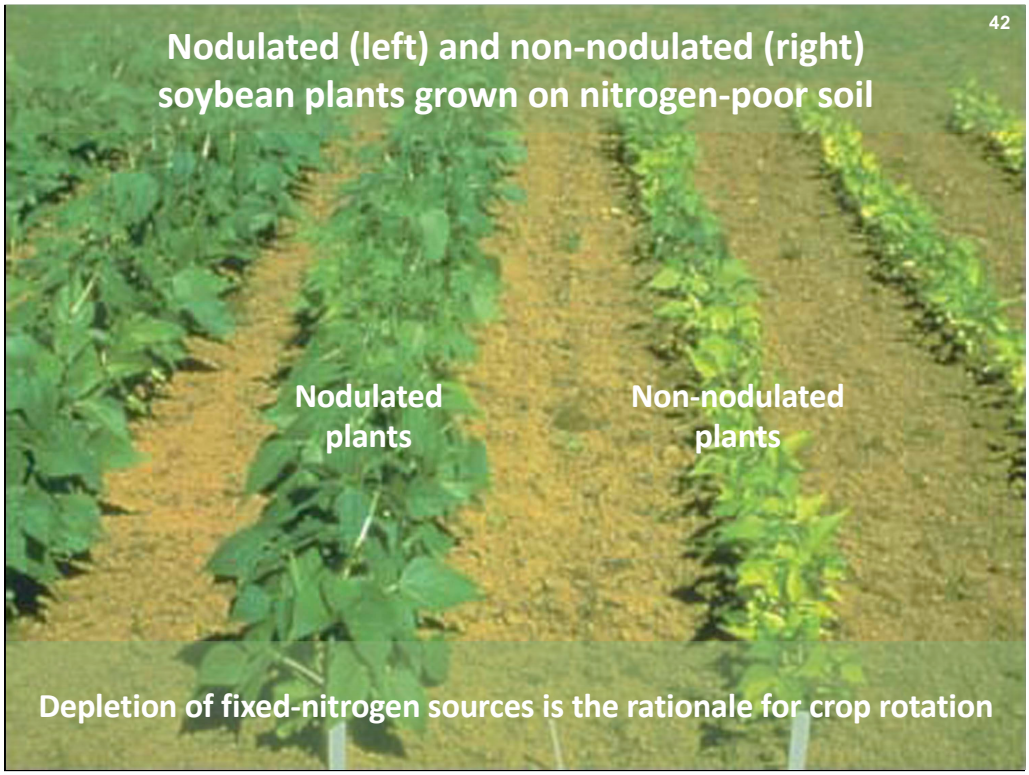
SOURCE: Fiore C.L., Jarett J.K., Olson N.D., Lesser M.P. (2010) Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends Microbiol.* 18(10): 455-463 PMID:20674366. Figure 3.

The most important abiotic factor affecting the internal environment of corals is light. In the presence of light, the zooxanthellae photosynthesize and produce hyperoxic conditions with strong gradients of oxygen within the tissues. Conversely, at night the respiratory activities of host and microbes create hypoxic or even anoxic conditions. These internal changes make it possible for different functional groups of symbiotic microbes - specifically the zooxanthellae involved in carbon-fixation (oxic conditions) and the cyanobacteria involved in nitrogen-fixation (hypoxic/anoxic conditions) to be both spatially and temporally separated over a range of conditions created by the coral itself.



SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019.

Figure 23.7. Soybean root nodules. The nodules develop from infection by the mutualistic symbiotic bacterium *Bradyrhizobium japonicum* (a species of rhizobia). The main stem of this soybean plant is about 0.5 cm in diameter.



SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019. Figure 23.8.

Effect of nodulation on plant growth. A field of nodulated (left) and non-nodulated (right) soybean plants growing in soil poor in fixed-nitrogen sources.

SOURCE: Gibson K.E., Kobayashi H., Walker G.C. (2008) Molecular determinants of a symbiotic chronic infection. *Annu. Rev. Genet.* 42: 413-441 PMID:18983260.

Rhizobial bacteria colonize legume roots for the purpose of biological nitrogen fixation. The rhizobium-legume symbiosis is established under fixed-nitrogen-limiting soil conditions and is estimated to contribute nearly half of all current biological nitrogen fixation. A complex series of events, coordinated by host and bacterial signal molecules, underlie the development of this symbiotic interaction. Rhizobia elicit *de novo* formation of a novel plant root organ within which they establish a chronic intracellular infection. Legumes permit rhizobia to invade these root tissues while exerting control over the infection process. Once rhizobia gain intracellular access to their host, legumes also strongly influence the process of bacterial differentiation that is required for nitrogen fixation. Symbiotic rhizobia play an active role in promoting their goal of host invasion and chronic persistence by producing a variety of signal molecules that elicit changes in host gene expression. In particular, rhizobia appear to promote their access to the host by producing a variety of signal molecules capable of suppressing a general pathogen defense response by the plant.

Steps in root nodule formation

(1) Recognition of correct partner by plant and bacteria (rhizobia)

(2) Attachment of the bacteria to the plant root hairs

(3) Secretion of nodulation (Nod) factors by bacteria

(4) Curling of root hairs and invasion by bacteria

(5) Spread of bacteria to main root via "infection thread"

(6) Differentiation of bacteria (some, not all) into "bacteroids"

(7) Competence for nitrogen (N_2) fixation in bacteroids

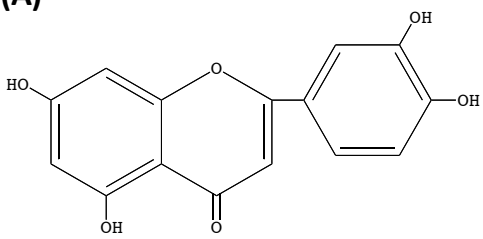
(8) Division of plant and bacterial cells

(9) Nodule formation

SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019. Figure 23.10.
Steps in the formation of a root nodule in a leguminous plant infected by *Rhizobium*. Formation of the bacteroid state is a prerequisite for nitrogen fixation. The time course of nodulation events from infection to effective nodule is about one month for soybeans.

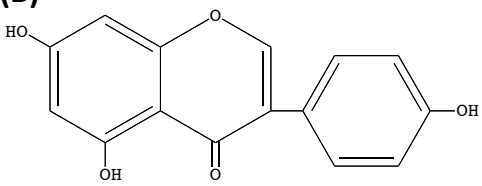
Plant flavonoids stimulate symbiont responses and determine the specificity of host-rhizobia symbiosis

(A)



Luteolin (5,7,3',4'-Tetrahydroxyflavone) **induces** *nod* gene expression in rhizobia (specifically, *Rhizobium leguminosarum*) produced by pea plants

(B)

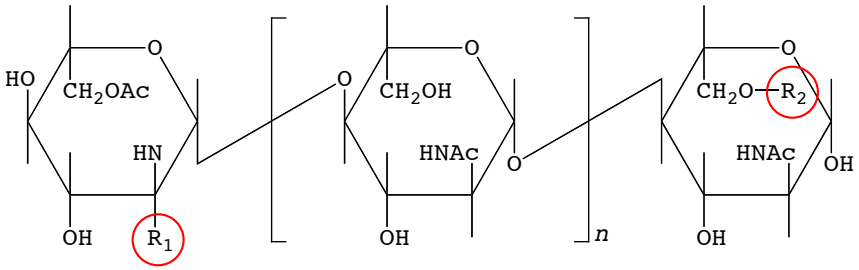


Genistein (5,7,4'-Trihydroxyisoflavone) **inhibits** *nod* gene expression in rhizobia (specifically, *Rhizobium leguminosarum*) produced by soybean plants

SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019. Figure 23.14.

Plant flavonoids and nodulation. Structures of flavonoid molecules that are **(A)** an inducer of *nod* gene expression and **(B)** an inhibitor of *nod* gene expression in *Rhizobium leguminosarum,* the species that nodulates pea plants. Note the similarities in the structures of the two molecules. **(A)** Structure of *luteolin*, a flavone derivative. It is produced by pea plants. **(B)** Structure of *genistein*, an isoflavone derivative. It is produced by soybean plants.

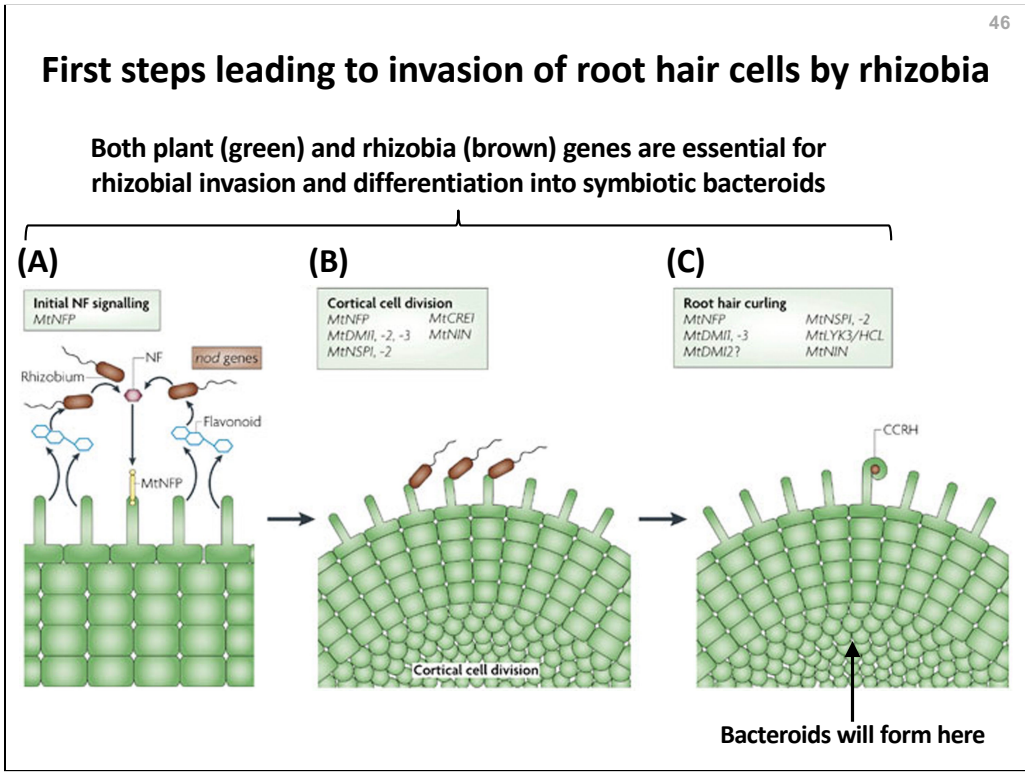
Rhizobial Nod factors stimulate host responses and determine the specificity of host-rhizobia symbiosis



Species	R_1	R_2
Rhizobium leguminosarum	-C18:1 or -C18:4	-H or -Ac
Sinorhizobium meliloti	-C16:2 or -C16:3	$-SO_4^{2-}$

SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019. Figure 23.12.

Nodulation (Nod) factors. General structure of the Nod factors produced by *Sinorhizobium meliloti* and *Rhizobium leguminosarum*, and the structural differences (R_1, R_2) that define the precise Nod factor of each species. The central hexose unit can repeat up to three times (*n* = 1-3). C16:2, palmitic acid with two double bonds; C16:3, palmitic acid with three double bonds; C18:1, oleic acid with one double bond; C18:4, oleic acid with four double bonds; Ac, acetyl.



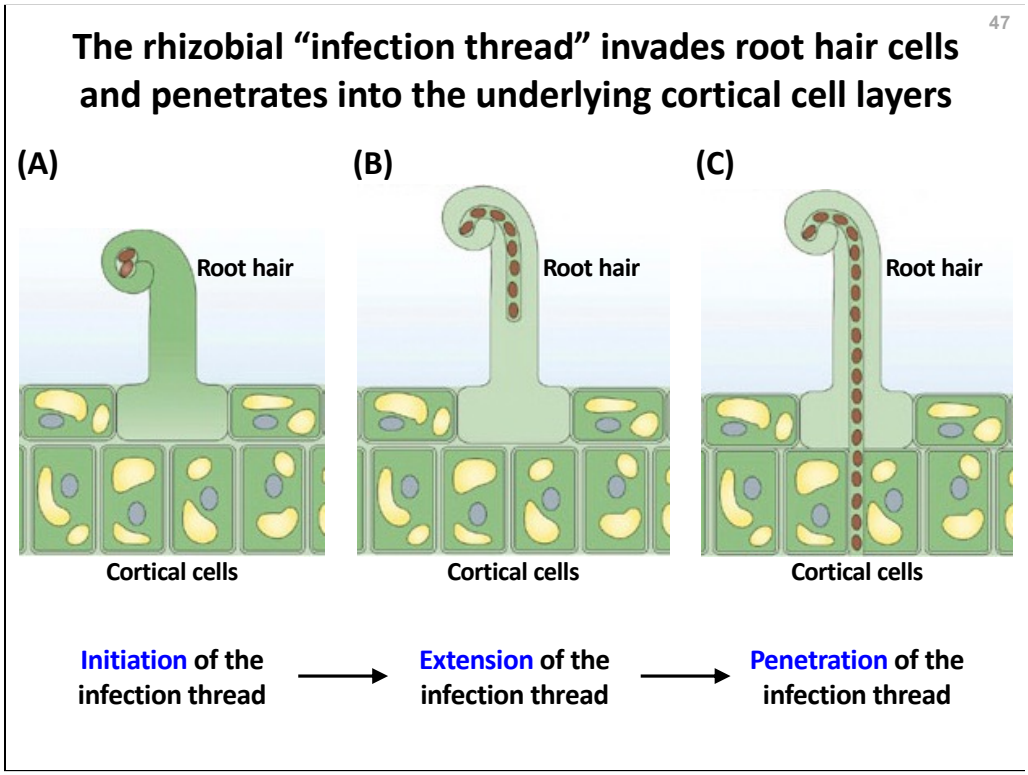
SOURCE: Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology* 5(8): 619-633 PMID:17632573. Figure 1.

The initial signaling dialogue between *Sinorhizobium meliloti* (rhizobial symbiont) and *Medicago truncatula* (plant host).

(A) Initial Nod factor (NF) signalling. The induction of rhizobial *nod* genes requires plant **flavonoids**. The *nod* gene products produce Nod factor (NF), which is initially perceived by the *M. truncatula* MtNFP receptor.

(B,C) Cortical cell divisions (B) and root hair curling (C) require many *M. truncatula* gene products; MtNFP; *MtDMI1*; *MtDMI2*; *MtDMI3*; *MtNSP1*; *MtNSP2*; *MtCRE1*; MtNIN. *MtLYK3/HCL* is required for colonized curled root hair (CCRH) formation, but not for the induction of cortical cell divisions.

The required rhizobial genes are boxed in brown. The required plant genes are boxed in light green. You do not need to memorize the names of any of these genes!!!



SOURCE: Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology* 5(8): 619-633 PMID:17632573. Figure 3.

Invasion of *Medicago trunculata* (plant host) root hairs by *Sinorhizobium meliloti* (bacterial symbiont).

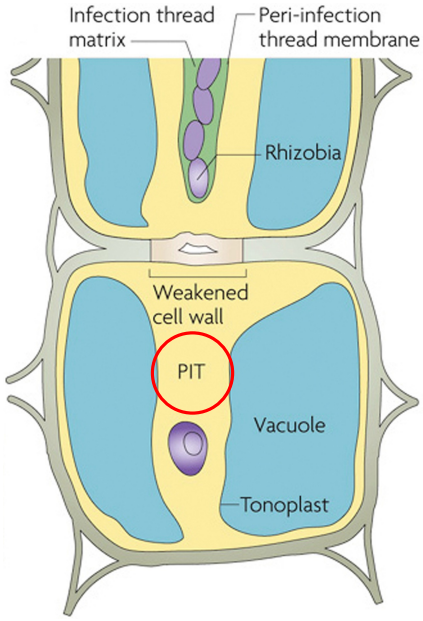
(A) Initiation of the infection thread. *S. meliloti* gene *exoY* is required. *M. trunculata* genes *MtLIN* and *MtNIN* are required.

(B) Extension of the infection thread to the base of the root hair cell. *S. meliloti* gene *exoH* gene is required. *M. trunculata* genes *MtNFP*, *MtLYK3/HCL*, *MtBIT1/ERN*, *MtNIN*, and *MtCRE1* are required.

(C) Penetration of the infection thread into the underlying cell layers. *M. trunculata* genes *MtCRE1*, *MtBIT1/ERN*, *MtRIT1*, and *MtSLI* are required.

The rhizobial “pre-infection thread” (PIT) forms ahead of the “infection thread” to prepare plant cells for invasion

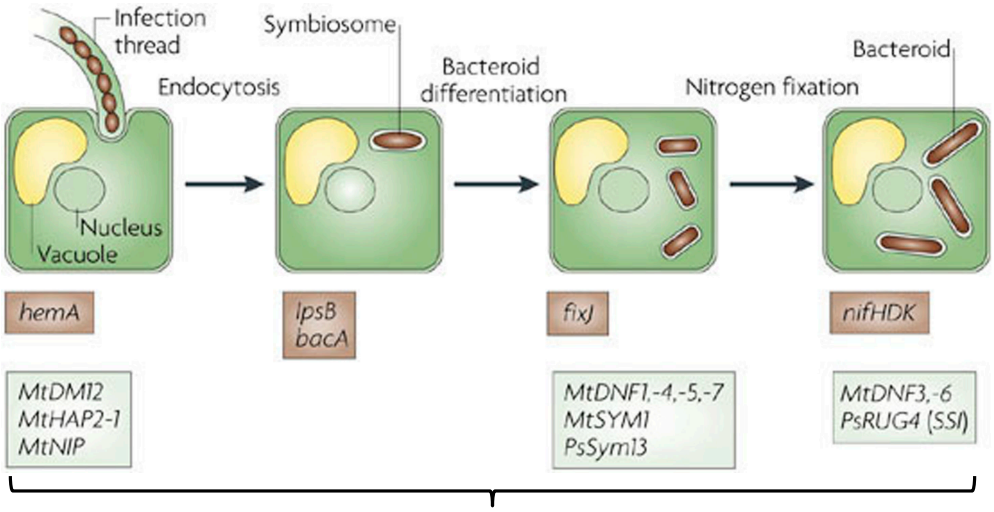
48



SOURCE: Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbiosis. *Nature Reviews Microbiology* 6(10): 763-775 PMID:18794914. Figure 5.

Intracellular accommodation structure in plant root nodule endosymbiosis involving rhizobia symbionts. A pre-infection thread (PIT) forms ahead of the bacteria-filled infection thread. The PIT can be induced by bacterial signals alone and contains an array of host-derived microtubules. The PIT is unique to the nodulating clade of rhizobia and is likely to have evolved from the pre-penetration apparatus of arbuscular mycorrhiza (fungal symbionts). A plant-derived perimicrobial membrane encloses the bacteria-filled infection thread and prevents microbial contact with the plant cytoplasm. This membrane synthesizes cell wall material, which contributes to the composition of the apoplastic interface between the symbiotic organisms.

Rhizobia invade plant cortical cells and differentiate into bacteroids within cytoplasmic "symbiosomes"



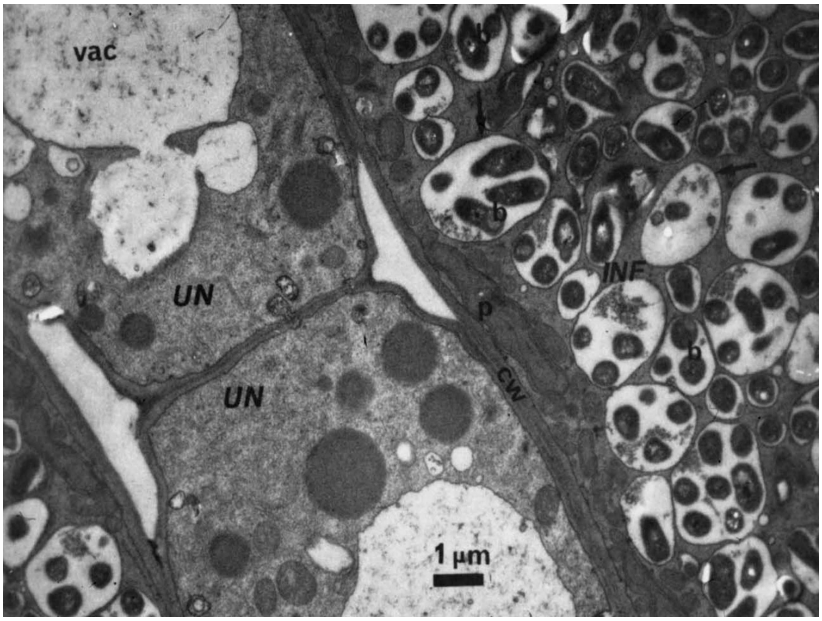
Both plant (green) and rhizobia (brown) genes are essential for rhizobial invasion and differentiation into symbiotic bacteroids

SOURCE: Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology* 5(8): 619-633 PMID:17632573. Figure 5.

Endocytosis of bacteria and bacteroid differentiation. Bacterial endocytosis requires the *Sinorhizobium meliloti hemA* gene, the *Medicago truncatula NIP* gene, and wild-type expression levels of the *M. truncatula MtDMI2* and *MtHAP2-1* genes. *S. meliloti lpsB* and *bacA* are required for bacterial survival within the symbiosome membrane. *S. meliloti fixJ*, *M. truncatula MtSYM1*, *MtDNF1,-4,-5,* and *-7*, and pea (*Pisum sativum) PsSYM13* are required for bacteroid differentiation. The *S. meliloti nifHDK* genes encode nitrogenase, which is the key enzyme for nitrogen fixation. The pea *PsRUG4* gene encodes sucrose synthase, which is required to support bacteroid nitrogen fixation. The *M. truncatula MtDNF3* and *-6* genes are required for the maintenance of nitrogen fixation.

The required rhizobial genes are boxed in brown. The required plant genes are boxed in light green.

Rhizobial bacteroids localize within “symbiosomes” (vesicular structures derived from plant membranes)



Source: http://www.google.ch/imgres?imgurl=http://web.mst.edu/~djwesten/Research_images/em.jpg&imgrefurl=http://web.mst.edu/~djwesten/Bj.html&h=896&w=1350&sz=118&tbnid=VZAeCF2pN_UgxM::&tbnh=100&tbnw=150&prev=/images%3Fq%3Dsymbiosome%2Bphoto&usg=__HeCSNyq9NYpMCpnL8btB9URwAb8=&ei=looRSpHpEYGO-AbvgOHWAw&sa=X&oi=image_result&resnum=3&ct=image

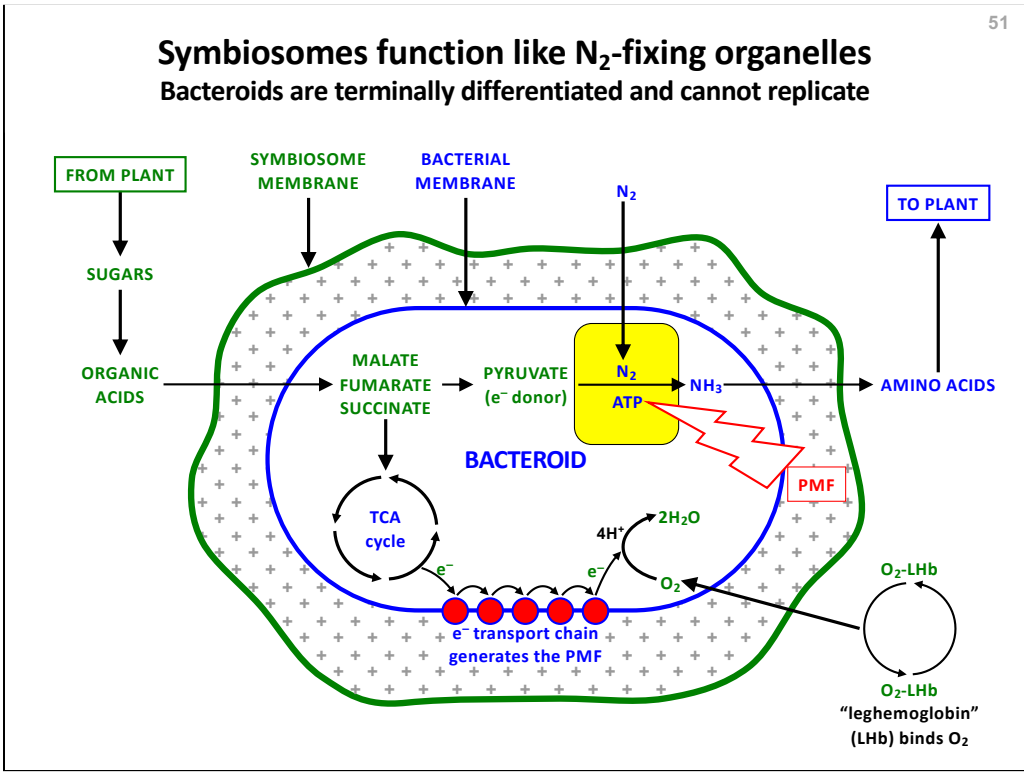
Bradyrhizobium japonicum is a Gram-negative, rod-shaped, nitrogen-fixing bacterium that develops a symbiosis with the soybean plant *Glycine max*. *B. japonicum* belongs to the family *Rhizobiaceae*, which includes other nitrogen-fixing bacteria that develop symbiosis with legumes. The bacterium "communicates" with the host plant and begins a process of plant and bacterial development that leads to a symbiotic partnership. The bacterium will attach to root hairs and release compounds that cause the root hairs to curl. Coordination of bacterial multiplication and inward-directed growth of the root hair results in formation of an infection thread (a tube derived from plant membranes). It is through this infection thread that the bacteria enter the cortical cells of the root and begin to colonize the developing root nodule.

Within the developing root nodule, bacteria divide and begin to differentiate into a bacteroid that is capable of fixing nitrogen. The term “bacteroid” refers to the bacterium existing in a symbiotic relationship to distinguish it from the free-living bacterium. As shown in the electron micrograph shown on this slide, the bacteroids are located inside a structure referred to as a symbiosome, which is derived from plant membrane. One to several bacteroids can be found in a single symbiosome. Therefore, nutrients must traverse multiple membranes to reach the bacteroids and fixed nitrogen must follow a similar complex path to reach the plant tissue. The nodule that results from this process is a highly specialized structure. It provides a physical barrier that keeps the free oxygen concentration low.

The plant cells within the nodule produce leghemoglobin, which serves as an oxygen carrier to the bacteria within the nodule. This enables the bacteria to obtain enough oxygen for respiration but ensures that the oxygen is in a bound form so that it cannot harm nitrogen-fixing enzymes inside the bacteria. Cutting open a nodule reveals the deep red color typical of leghemoglobin when it binds oxygen.

What do the individual partners in this symbiosis get from each other? The plant provides the bacterium with a "safe" environment and a steady supply of fixed carbon for energy and growth. This carbon source is referred to as “photosynthate”, as it is derived from the product of photosynthesis. In most rhizobium/legume symbiosis, photosynthate refers to the dicarboxylic acids succinate, fumarate, and malate. In return, the bacteria provide the plant with fixed nitrogen, i.e., dinitrogen (N_2) gas that has been reduced and converted into a form (amino acids) readily utilized by the plant. The result of this symbiosis is a dramatic increase in plant production without the need for adding external

fertilizer.



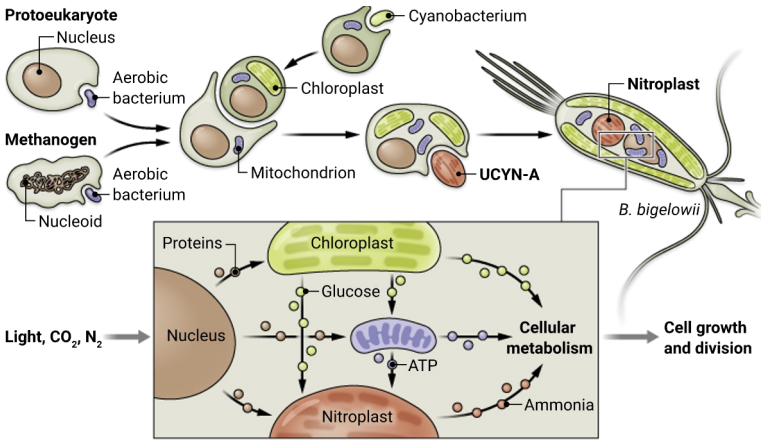
SOURCE: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019. Figure 23.15.

The root nodule bacteroid. Schematic diagram of major metabolic reactions and nutrient exchanges in the bacteroid. The symbiosome is a collection of bacteroids surrounded by a single membrane originating from the plant.

Nitrogen fixation requires the enzyme nitrogenase. Nitrogenase from bacteroids shows the same biochemical properties as the enzyme from free-living N_2-fixing bacteria, including O_2 sensitivity and the ability to reduce acetylene as well as N_2. Bacteroids are dependent on the plant to provide the electron donor for N_2 fixation. The major organic compounds transported across the symbiosome membrane and into the bacteroid proper are citric acid cycle intermediates - in particular, the C_4 organic acids succinate, malate, and fumarate. These molecules are used as electron donors for ATP production and, following conversion to pyruvate, as the source of electrons for reduction of N_2 to ammonia (NH_3).

The product of N_2 fixation is ammonia (NH_3)., and the plant assimilates most of this ammonia by forming organic nitrogen compounds, such as amino acids. The ammonia-assimilating enzyme glutamine synthetase is present in high levels in the plant cell cytoplasm and converts glutamate and ammonia into glutamine. Glutamine and a few other organic nitrogen compounds transport fixed nitrogen throughout the plant.

The “nitroplast”: a nitrogen-fixing organelle (2024)

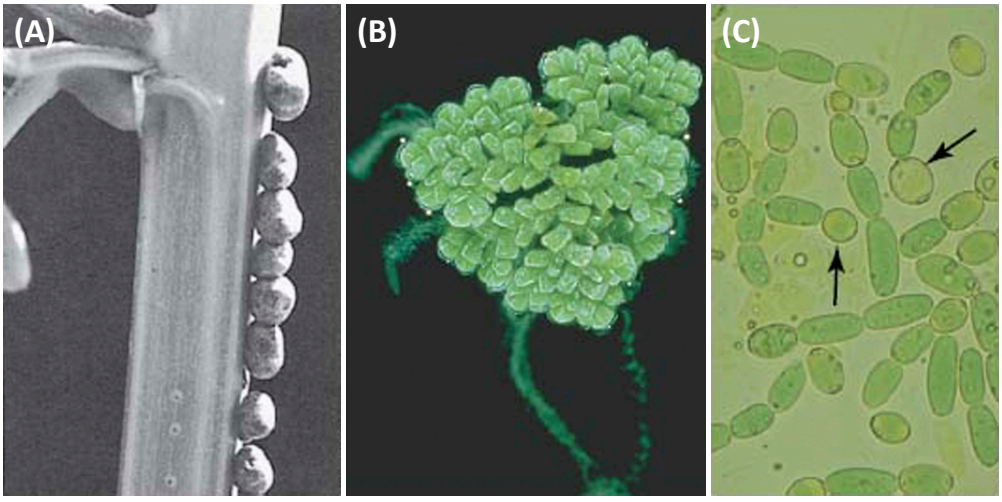


Source: Massana R (2024) The nitroplast: a nitrogen-fixing organelle. *Science* 384(6692): 160-161 PMID: 38603513.

Source: Coale TH, Loconte V, Turk-Kubo KA, Vanslembrouck B, Mak WKE, Cheung S, Ekman A, Chen JH, Hagino K, Takano Y, Nishimura T, Adachi M, Le Gros M, Larabell C, Zehr JP (2024) Nitrogen-fixing organelle in a marine alga. *Science* 384(6692): 217-222 PMID: 38603509

Evolution and function of the nitroplast. Multiple organelles in eukaryotic cells, including mitochondria, chloroplasts, and nitroplasts, evolved from the integration of endosymbiotic bacteria. In *Braarudosphaera bigelowii*, the chloroplast fixes inorganic carbon to produce glucose, which feeds the respiratory chain in mitochondria that produces adenosine triphosphate (ATP), which in turn fuels nitrogen fixation in the nitroplast. Glucose, ammonia, and ATP generated by the organelles, together with externally incorporated compounds (phosphorous, mineral nutrients, and vitamins), are the building blocks for cell metabolism, resulting in cell growth and division.

Nitrogen fixation: more than just root nodules…



Source: *Brock Biology of Microorganisms [15th edition for Kindle]*, Chapter 23: Microbial Symbioses with Microbes, Plants, and Animals (pp. 732-764), published by Pearson Education Inc., San Francisco © 2019. Figure 23.16 and Figure 23.17.

(A) Stem nodules formed by stem-nodulating *Azorhizobium*. The photograph shows the stem of the tropical legume *Sesbania rostrata*. On the left side of the stem are uninoculated sites, on the right identical sites inoculated with stem-nodulating rhizobia.

(B) *Azolla-Anabaena* symbiosis. Intact association showing a single plant of *Azolla pinnata*. The diameter of the plant is approximately 1 cm.

(C) *Azolla-Anabaena* symbiosis. Cyanobacterial symbiont *Anabaena azollae* as observed in crushed leaves of *A. pinnata*. Single cells of *A. azollae* are about 5 µm wide. Note the spherical-shaped heterocysts (lighter color, arrows), the site of nitrogen fixation in the cyanobacterium.