Biological Nitrogen Fixation

Conversion of dinitrogen gas (N_2) to ammonia (NH_3)

Availability of fixed N often factor most limiting to plant growth

N-fixation ability limited to few bacteria, either as free-living organisms or in symbiosis with higher plants

First attempt to increase forest growth through N-fixation in Lithuania, 1894 (lupines in Scots pine)

Biological nitrogen fixation:

$$N_2 + 8\ \text{flavodoxin}^- + 8H^+ + 16\ MgATP^{2-} + 18\ H_2O \xrightarrow{\text{nitrogenase}} 2NH_4^+ + 2OH^- + 8\ \text{flavodoxin} + 16\ MgADP^- + 16H_2PO_4^- + H_2$$

1. Rare, extremely energy consuming conversion because of stability of triply bonded N_2
2. Produces fixed N which can be directly assimilated into N containing biomolecules

Ecology of nitrogen-fixing bacteria

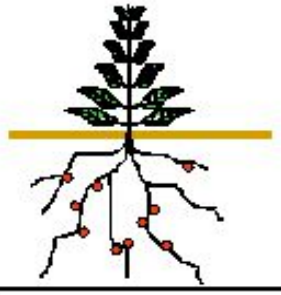
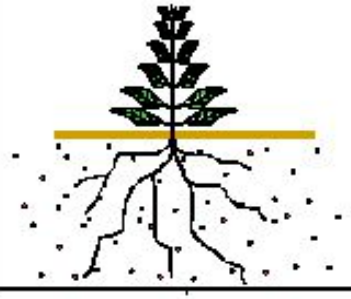
System of N_2 fixation (and microbes involved) ($N_2 \rightarrow NH_3$)	SYMBIOSIS (e.g. *Rhizobium*)	ASSOCIATION (e.g. *Azospirillum*)	FREE-LIVING (e.g. *Rhodospiorillum*)	
				
Energy source (Organic C)	Sucrose from the host plant	Root exudates from the host plant	Heterotroph (plant residues)	Autotroph (photo-synthesis)
Estimates of fixation rate (kg N/ha/y)	50-400	10-200	1-2	10-80

Tableau I. **Une sélection de quelques bactéries fixatrices d'azote.**

Groupes phylogéniques, nombre de fixateurs caractérisés, exemples	Métabolisme énergétique, tension d'oxygène compatible avec la fixation de l'azote, interaction avec les plantes
Bactéries vertes sulfureuses	
4 genres, 6 espèces	
Chlorobium limicola	PAT Anaérobiose
Firmibactéries (Gram+)	
3 genres, 22 espèces	
Bacillus polymixa	CHT Microaérobiose
Clostridium acetobutylicum	CHT Anaérobiose
Clostridium pasteurianum	CHT Anaérobiose
Thallobactéries (Gram+)	
4 genres, x espèces	
Arthrobacter sp	CHT Microaérobiose
Frankia	CHT Microaérobiose. Symbiote actinorhizien (pe aulne, casuarina)
Héliobactéries	
3 genres, 3 espèces	
Heliobacterium chlorum	PHT Anaérobiose
Heliospirillum gestii	PHT Anaérobiose
Cyanobactéries	
14 genres, x espèces	
Anabaena 7120	PAT Aérobiose
Anabaena azollae	PAT Aérobiose. Symbiote de la fougère *Azolla*
Nostoc 73102	PAT Aérobiose
Gloeothece 6501	PAT Microaérobiose
Campylobactéries	
1 genre, 1 espèce	
Protéobactéries α	
20 genres, 54 espèces	
Acetobacter diazotrophicus	CHT Microaérobiose. Endophyte de la canne à sucre
Azorhizobium caulinodans	CHT Microaérobiose. Symbiote de *Sesbania rostrata*
Azospirillum brasilense	CHT Microaérobiose. Associé aux racines des Graminées
Bradyrhizobium japonicum	CHT Microaérobiose. Symbiote du soja
Rhizobium leguminosarum	CHT Microaérobiose. Symbiote du pois
Rhizobium meliloti	CHT Microaérobiose. Symbiote de la luzerne
Rhodobacter capsulatus	PHT Anaérobiose
Rhodospirillum rubrum	PHT Anaérobiose
Protéobactéries β	
7 genres, 11 espèces	
Alcaligenes faecalis	CHT Microaérobiose. Associé aux racines du riz
Azoarcus spp	CHT Microaérobiose. Endophyte de l'herbe de Kallar *(Leptochloa fusca)*
Derxia gummosa	CHT Microaérobiose
Herbaspirillum seropedicae	CHT Microaérobiose. Endophyte de la canne à sucre
Thiobacillus ferrooxidans	CAT Microaérobiose
Protéobactéries γ	
18 genres, 44 espèces	
Azotobacter vinelandii	CHT Aérobiose
Beggiatoa alba	CAT Microaérobiose
Enterobacter agglomerans	CHT Anaérobiose
Klebsiella pneumoniae	CHT Anaérobiose
Pseudomonas stutzeri	CHT Microaérobiose
Protéobactéries δ	
2 genres, 10 espèces	
Desulfovibrio gigas	CHT Anaérobiose
Archaebactéries	
4 genres, 7 espèces	
Methanobacterium ivanovii	CAT Anaérobiose
Methanococcus thermolithotrophicus	CAT Anaérobiose

CAT : chimioautotrophe ; CHT : chimiohétérotrophe ; PAT : photoautotrophe ; PHT : photohétérotrophe.

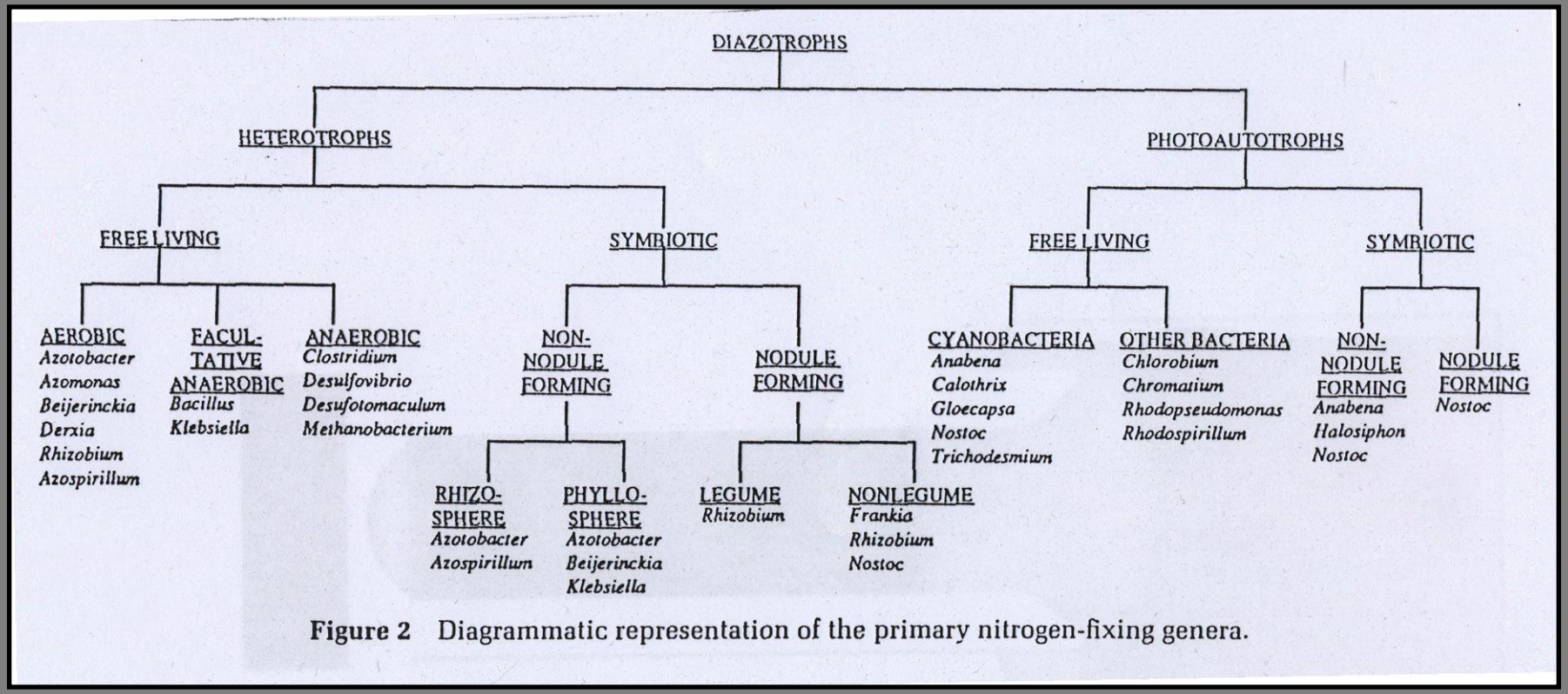


Figure 2 Diagrammatic representation of the primary nitrogen-fixing genera.

N-fixation requires energy input:

- Reduction reaction, e^- must be added (sensitive to O_2)
- Requires ~35 kJ of energy per mol of N fixed (theoretically)
- Actual cost: ~15-30g CH per g of NH_3 produced
- Assimilation of NH_3 into organic form takes 3.1-3.6 g CH

Enzymology of N fixation

Only occurs in certain prokaryotes

- **Rhizobia fix nitrogen in symbiotic association with leguminous plants**
- **Rhizobia fix N for the plant and plant provides Rhizobia with carbon substrates**
- **All nitrogen fixing systems appear to be identical**
- **They require nitrogenase, a reductant (reduced ferredoxin), ATP, O-free conditions and regulatory controls (ADP inhibits and NH_4^+ inhibits expression of nif genes**

Biological nitrogen fixation is the reduction of atmospheric nitrogen gas (N_2) to ammonium ions (NH_4^+) by the oxygen-sensitive enzyme, **nitrogenase**.
Reducing power is provided by NAPH/ferredoxin, via an Fe/Mocentre.

Plant genomes lack any genes encoding this enzyme, which occurs only in prokaryotes (bacteria).

Even within the bacteria, only certain free-living bacteria (Klebsiella, Azospirillum, Azotobacter), blue-green bacteria (Anabaena) and a few symbiotic Rhizobial species are known nitrogen-fixers.

Another nitrogen-fixing association exists between an Actinomycete (Frankia spp.) and alder (Alnus spp.)

The enzyme **nitrogenase** catalyses the conversion of atmospheric, gaseous dinitrogen (N_2) and dihydrogen (H_2) to ammonia (NH_3), as shown in the chemical equation below:

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

The above reaction seems simple enough and the atmosphere is 78% N_2, so <u>why is this enzyme so important</u>?

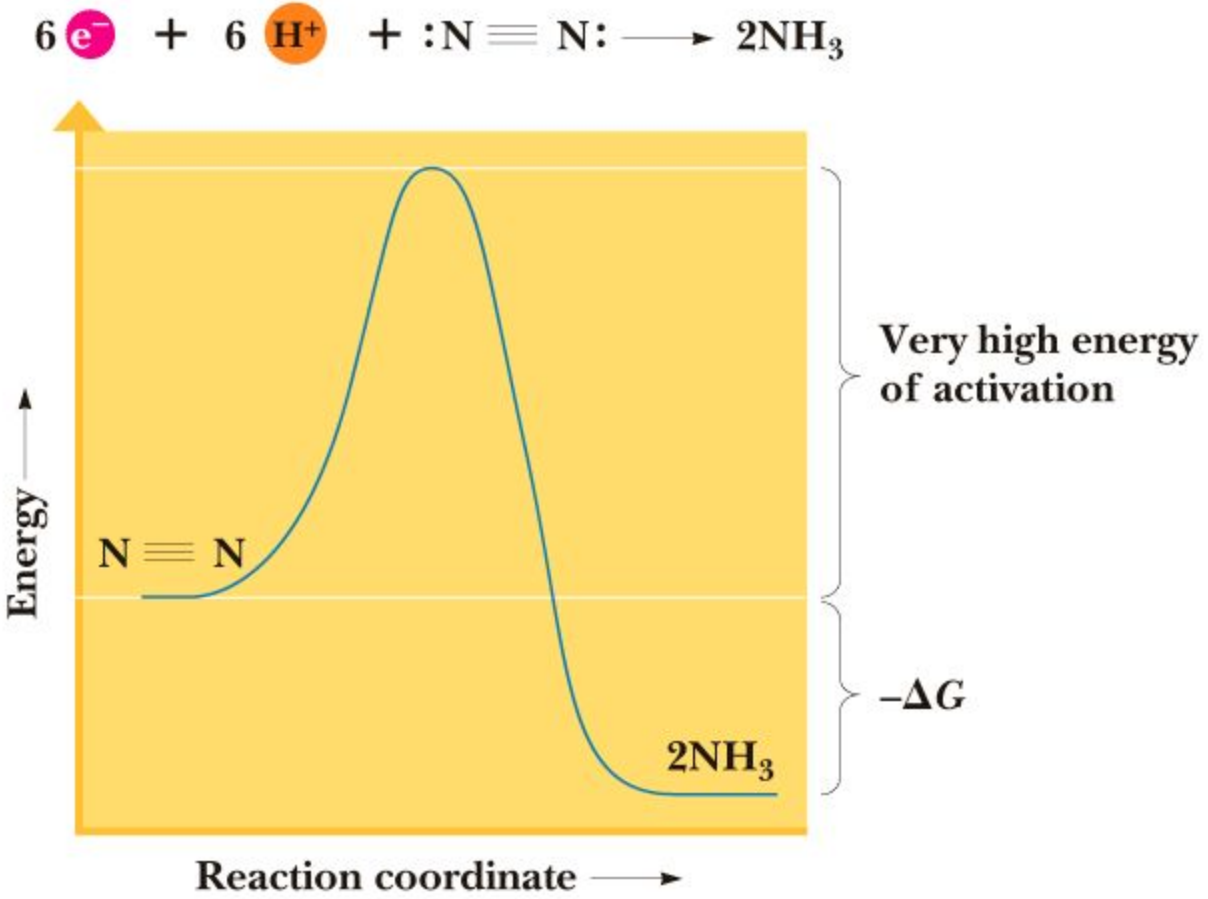
The incredibly strong (triple) bond in N_2 makes this reaction very difficult to carry out efficiently.
In fact, nitrogenase consumes ~16 moles of ATP for every molecule of N_2 it reduces to NH_3, which makes it one of the most energy-expensive processes known in Nature.

Nitrogenase Complex

Two protein components: nitrogenase reductase and nitrogenase

- Nitrogenase reductase is a 60 kD homodimer with a single 4Fe-4S cluster
- Very oxygen-sensitive
- Binds MgATP
- 4ATP required per pair of electrons transferred
- Reduction of N_2 to $2NH_3 + H_2$ requires 4 pairs of electrons, so **16 ATP are consumed per N_2**

Garrett & Grisham: Biochemistry, 2/e
Figure 26.4



Why should nitrogenase need ATP???

- N_2 reduction to ammonia is thermodynamically favorable
- However, the activation barrier for breaking the N-N triple bond is enormous
- **16 ATP** provide the needed activation energy

Nitrogenase

A 220 kD heterotetramer

- Each molecule of enzyme contains 2 Mo, 32 Fe, 30 equivalents of acid-labile sulfide (FeS clusters, etc)
- Four 4Fe-4S clusters plus two FeMoCo, an iron-molybdenum cofactor
- Nitrogenase is **slow - 12 e^- pairs per second, i.e., only three molecules of N_2 per second**

Genetic Clusters

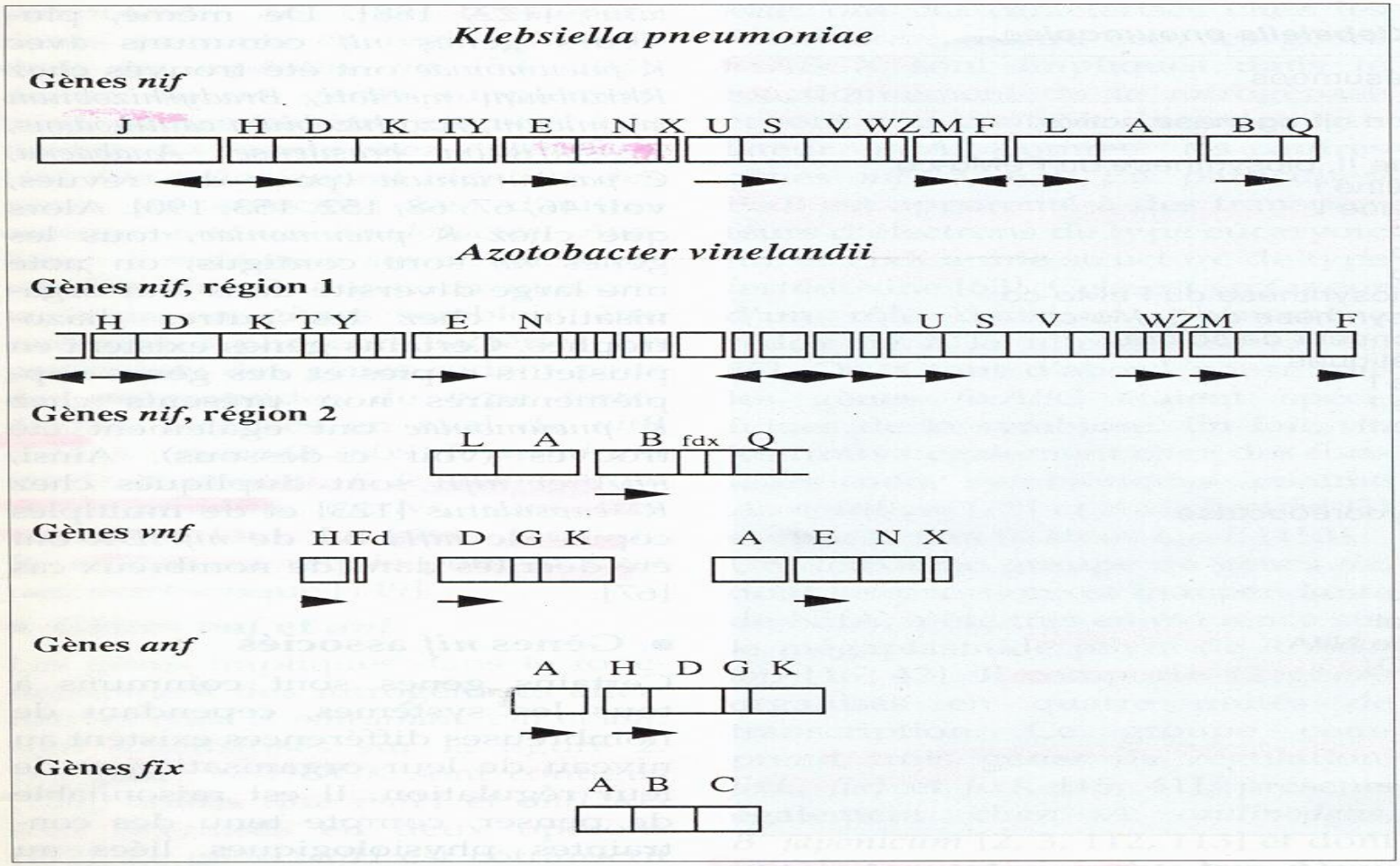
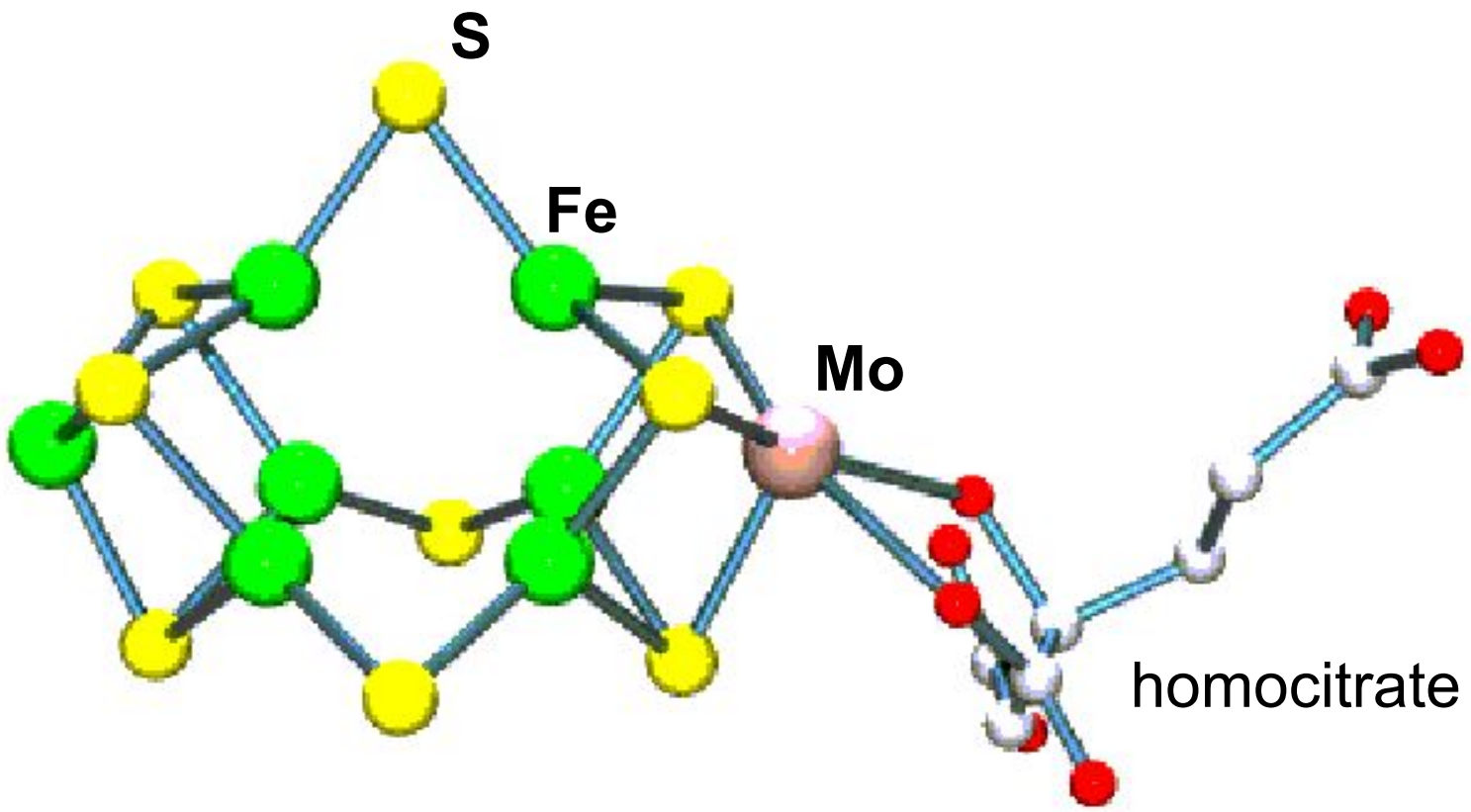


Fig 2. Organisation des gènes de la fixation de l'azote de Klebsiella pneumoniae *et d'*Azotobacter vinelandii. *Les gènes contigus correspondent à des opérons polycistroniques. Les flèches indiquent le sens de transcription à partir de promoteurs dépendant du facteur* σ^{54}.

The genes and products

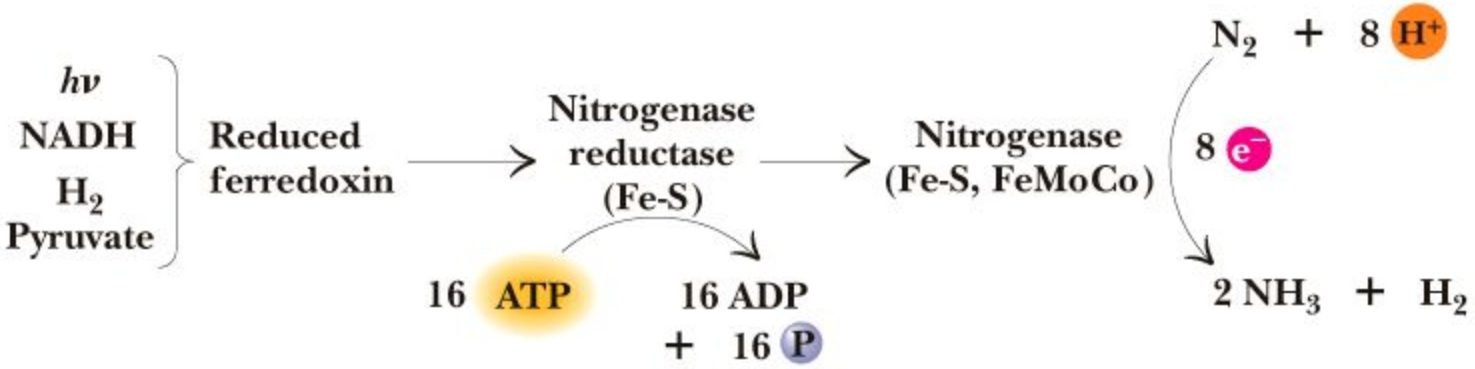
Tableau II. **Fonction des gènes *nif* de *Klebsiella pneumoniae*.**

Gènes	Fonctions établies ou présumées
1. Gènes impliqués dans la synthèse d'une nitrogénase active	
nifH	Polypeptide de la protéine II, biosynthèse du FeMo-co
nifD	Polypeptide α de la protéine I
nifK	Polypeptide β de la protéine I
nifE	Biosynthèse du FeMo-co
nifN	Biosynthèse du FeMo-co
nifB	Biosynthèse du FeMo-co
nifV	Homocitrate synthase, biosynthèse du FeMo-co
nifQ	Métabolisme du Mo, biosynthèse du FeMo-co
nifS	Cystéine désulfurase, donneur de soufre pour les groupes prosthétiques
nifW	Maturation de la protéine I
nifZ	Maturation de la protéine I
nifM	Maturation de la protéine II
2. Transport des électrons	
nifJ	Pyruvate-flavodoxine oxydoréductase
nifF	Flavodoxine
3. Régulation	
nifA	Activateur transcriptionnel
nifL	Modulateur de l'activité de NifA en présence de NH_3 ou O_2
4. Gènes non essentiels	
nifT	Inconnu
nifY	Inconnu
nifX	Inconnu
nifU	inconnu



Fe - S - Mo electron transfer cofactor in nitrogenase

Garrett & Grisham: Biochemistry, 2/e
Figure 26.6



Three Types of N-fixers Important in Forest Soils

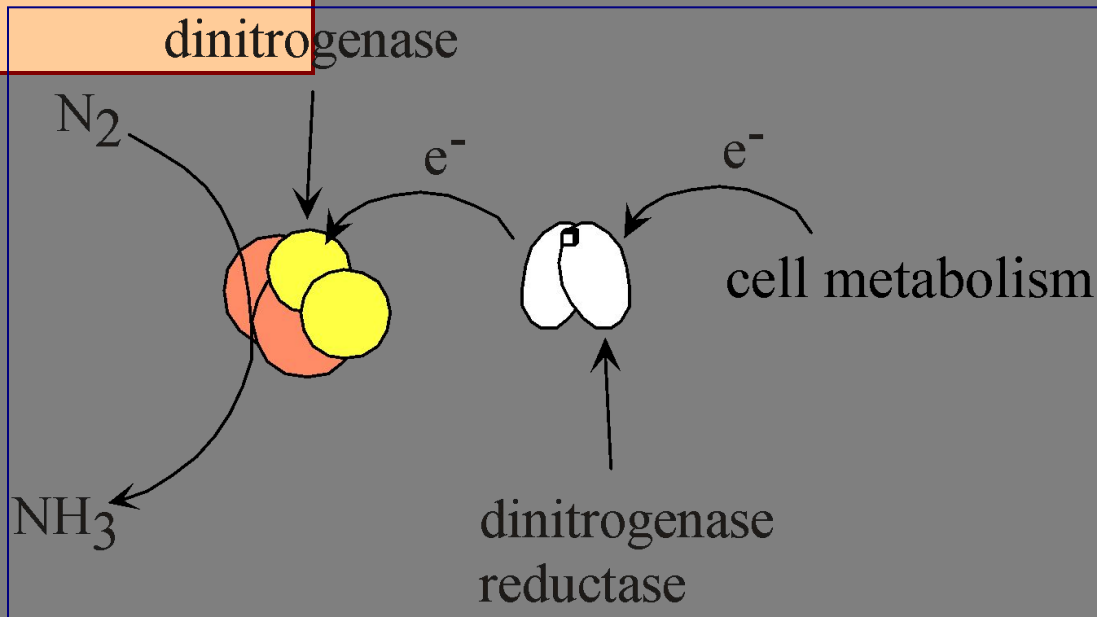
Cyanobacteria: Autotrophic N-fixers, protect nitrogenase with specialized *heterocyst* cells.

Heterotorophic bacteria: Free-living or associative with rhizosphere. Use energy from decomposing organic matter to fix N, protect nitrogenase by rapidly converting O_2 to CO_2 through respiration.

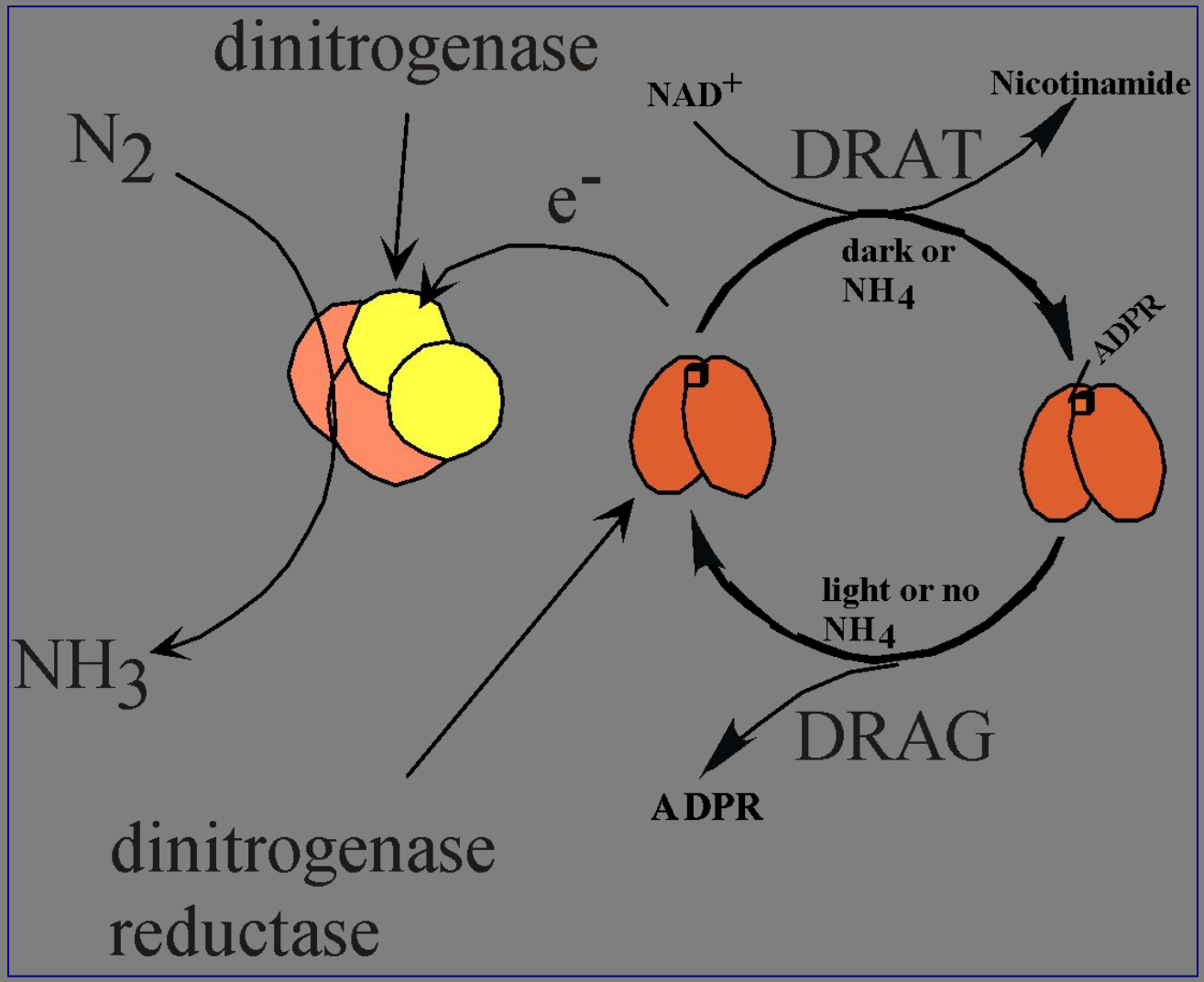
Symbiotic bacteria: Plants form nodules to house bacteria and provide C as energy source (*Rhizobium*/*Bradyrhizobium* for legumes, *Frankia* for non-legumes). Nodules contain a form of hemoglobin which binds O_2, protecting nitrogenase enzyme.

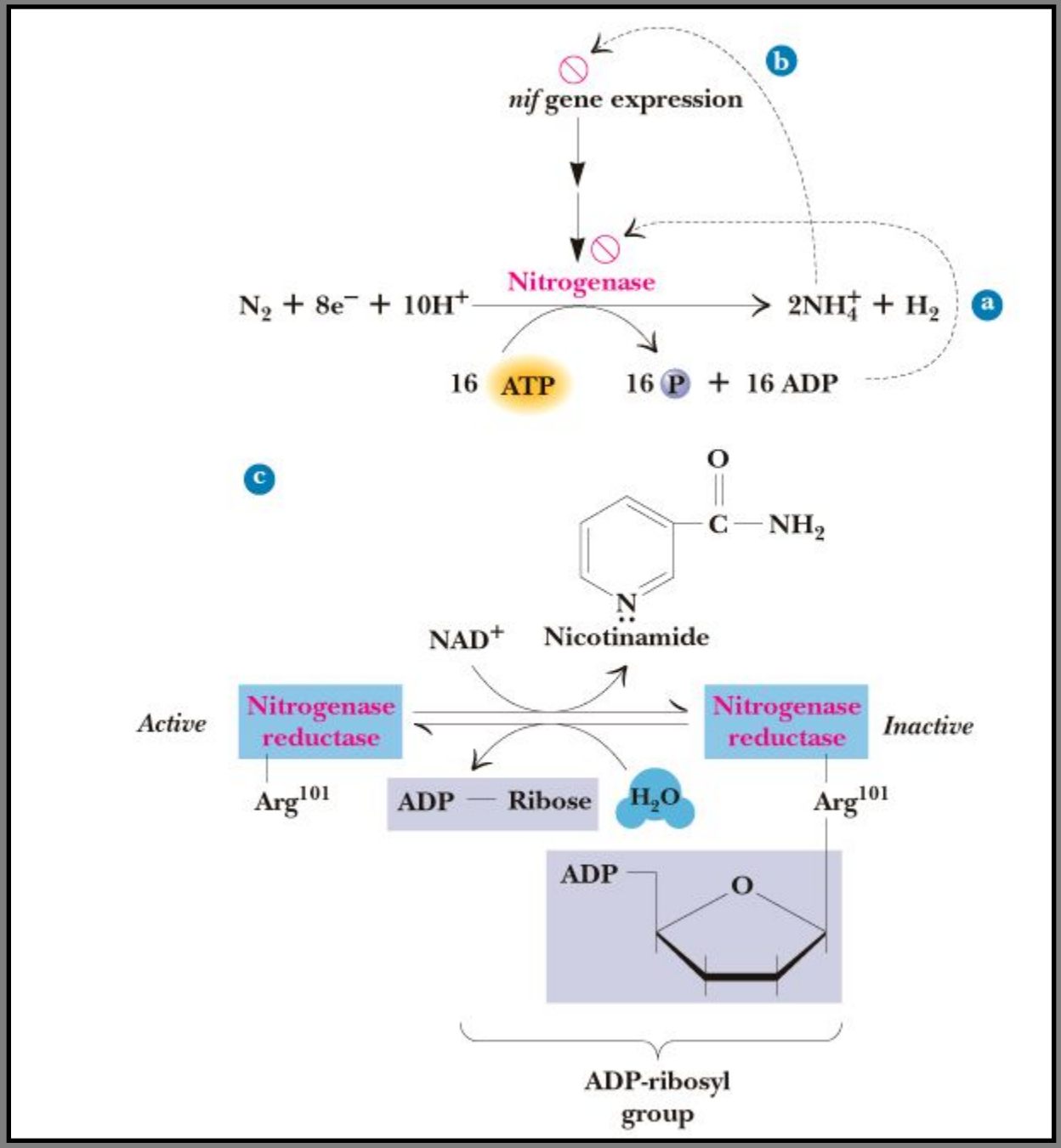
Nitrogen fixation in Klebsiella

- **Nif system is turned on when**
  - **No fixed nitrogen**
  - **Anaerobic**
  - **Temperature below 30°C**
- **Nitrogenase is made**
  - **Converts N_2 to NH_3**



ADP ribosylation of dinitrogenase reductase




b
nif gene expression
Nitrogenase
$N_2 + 8e^- + 10H^+$
$2NH_4^+ + H_2$
a
16 ATP
16 P + 16 ADP
c
O
C — NH_2
N
Nicotinamide
NAD^+
Active
Nitrogenase reductase
Arg101
ADP — Ribose
H_2O
Nitrogenase reductase
Inactive
Arg101
ADP
O
ADP-ribosyl group

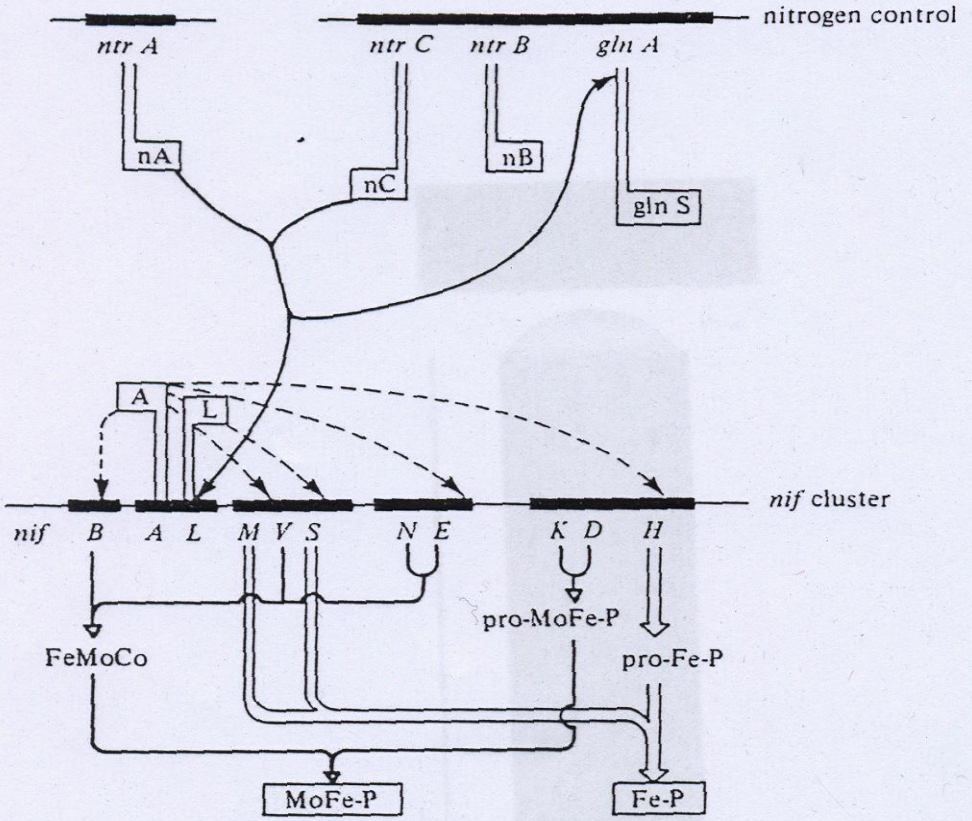


Figure 10.5. Organization and control of the *nif* cluster in *K. pneumoniae*. The situation under the condition of derepression is shown. The products of *ntrA* + *ntrC*, designated as nA and nC, together activate the promotor of *glnA* and of *nifA-nifL*. The *nifA* product (A) then activates the remaining promotors of the *nif* cluster. pro-Fe-P, Polypeptide of azoferredoxin; incorporation of the iron-sulfur centers is controlled by *nifM* and *nifS* products; pro-MoFe-P, precursor of molybdoferredoxin; the iron-molybdenum cofactor results from the products of genes *nifB*, *nifN*, *nifV*, and *nifE*. In the presence of ammonia product nB prevents activation at the nitrogen control level and product L at the *nif* cluster level. Solid arrows indicate the promotors at which transcription is derepressed by the gene products nA + nC. Dotted arrows indicate the promotors at which transcription is derepressed by the gene product A.

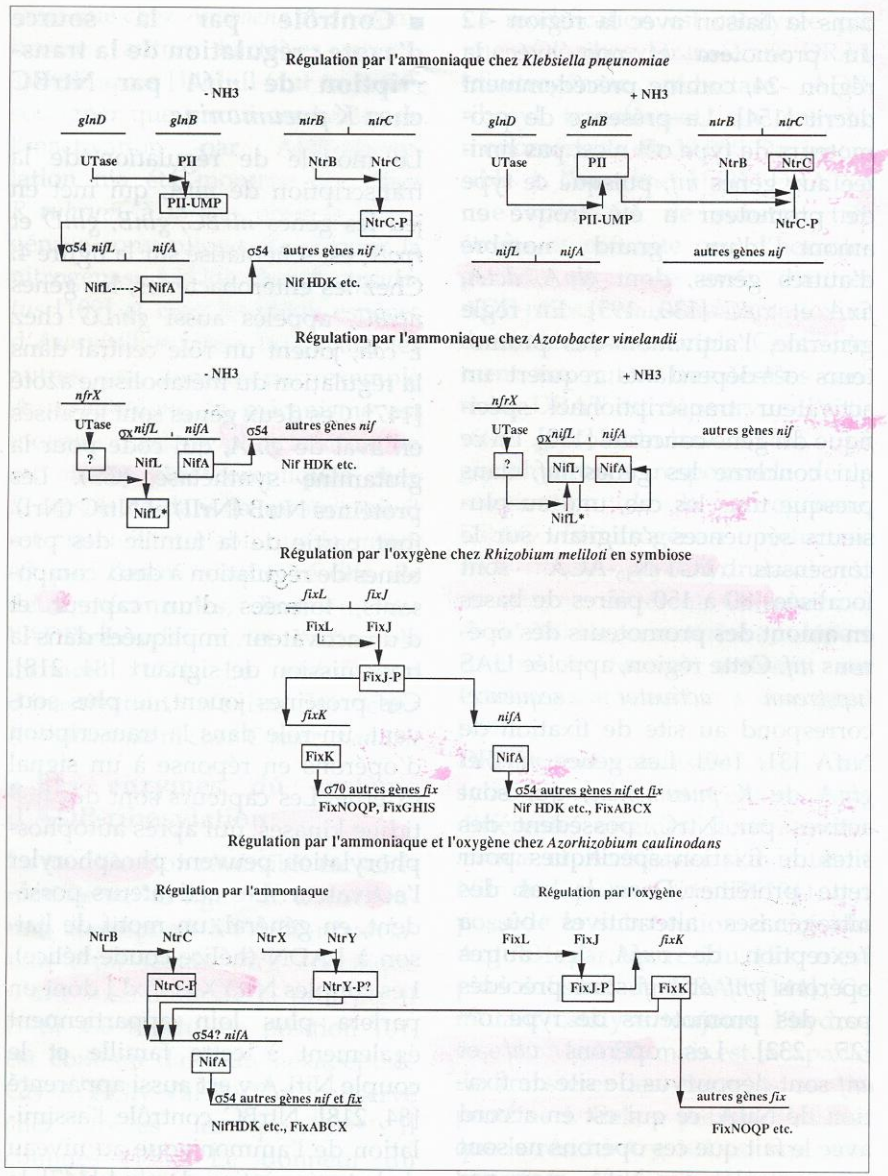


Fig 4. Quelques modèles de régulation chez différentes bactéries fixatrices d'azote. K pneumoniae *fixe l'azote en anaérobiose et en absence d'ammoniaque,* A vinelandii *dans l'air et en absence d'ammoniaque,* R meliloti *seulement en symbiose,* A caulinodans *en symbiose et à l'état libre en microaérobiose en absence d'ammoniaque. Le modèle présenté pour* A caulinodans *correspond aux conditions de fixation à l'état libre. Les principales protéines régulatrices sont encadrées.*

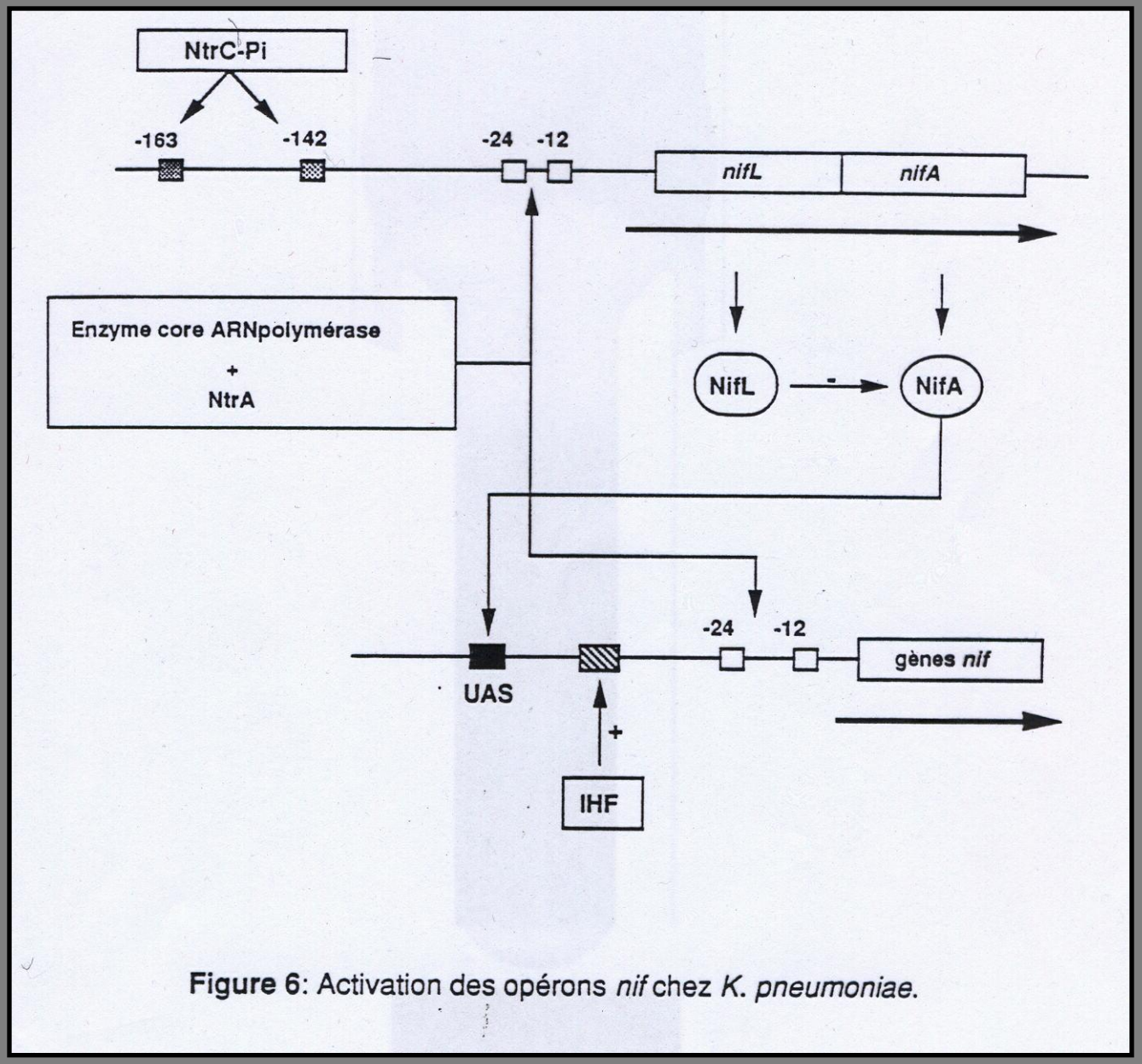
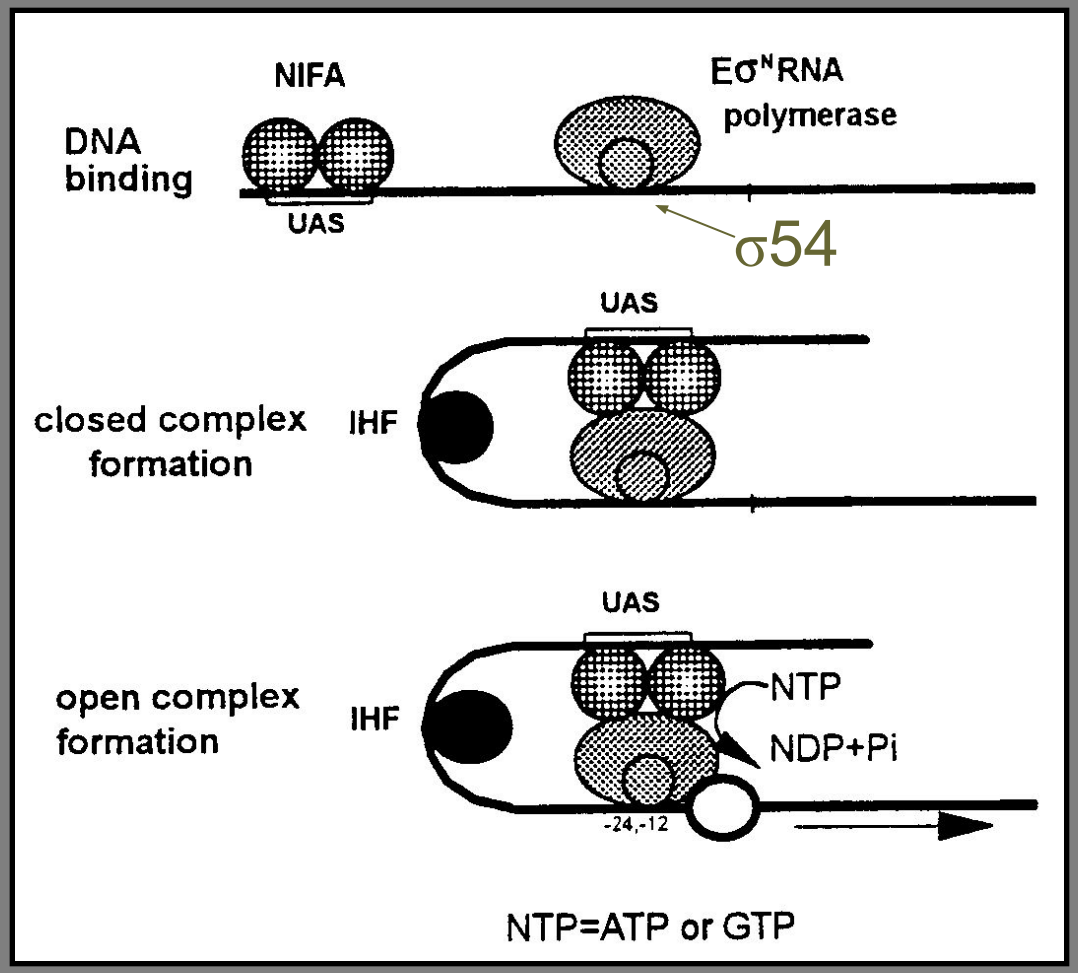
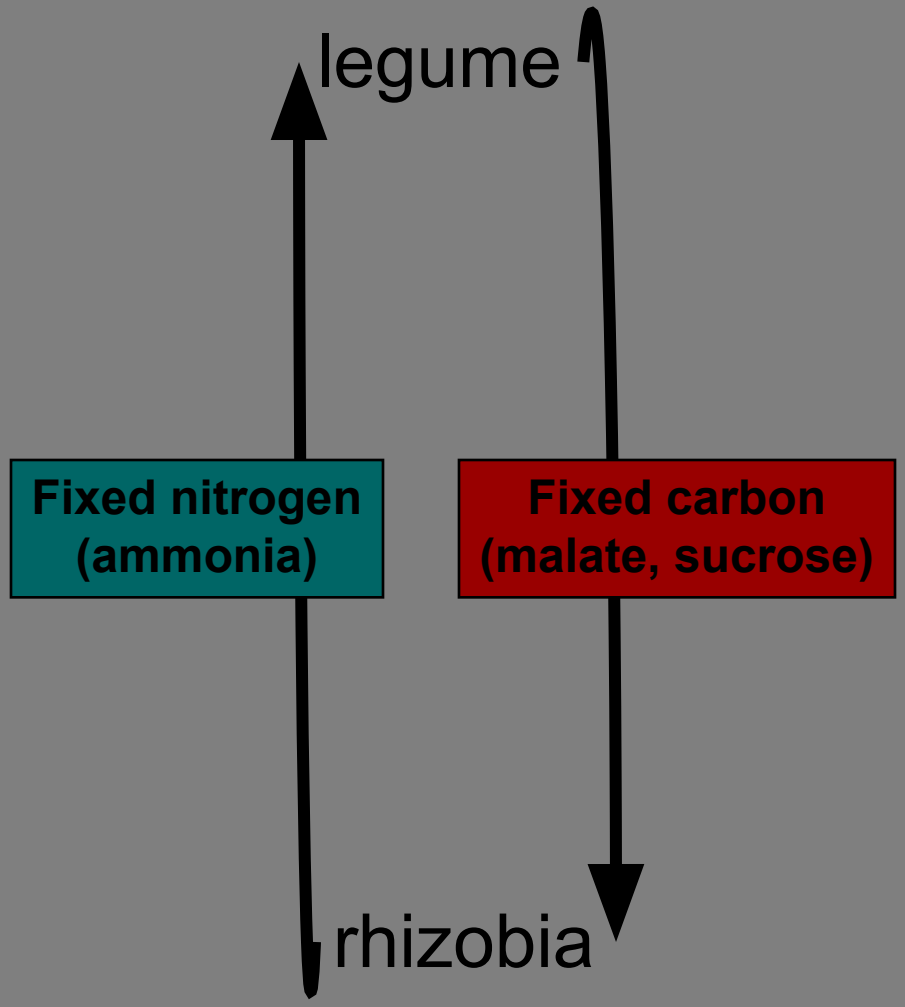


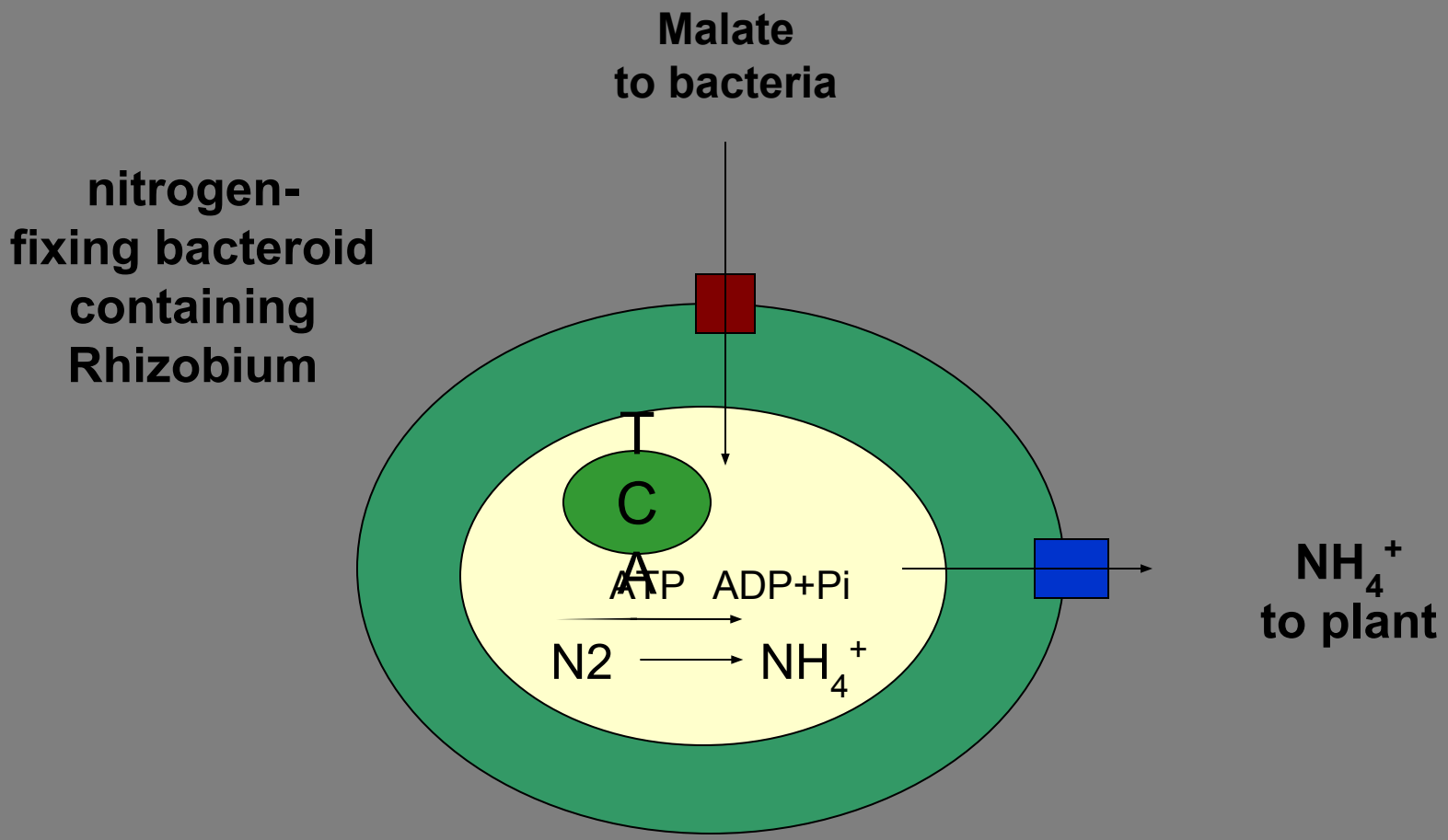
Figure 6: Activation des opérons *nif* chez *K. pneumoniae*.

Activation of nif promoters by NifA:
A mechanism similar to RNAP(σ54) activation by NtrC




legume
Fixed nitrogen
(ammonia)
Fixed carbon
(malate, sucrose)
rhizobia

Exchange of nutrients during Rhizobium-legume symbiosis



Symbiotic Nitrogen Fixation

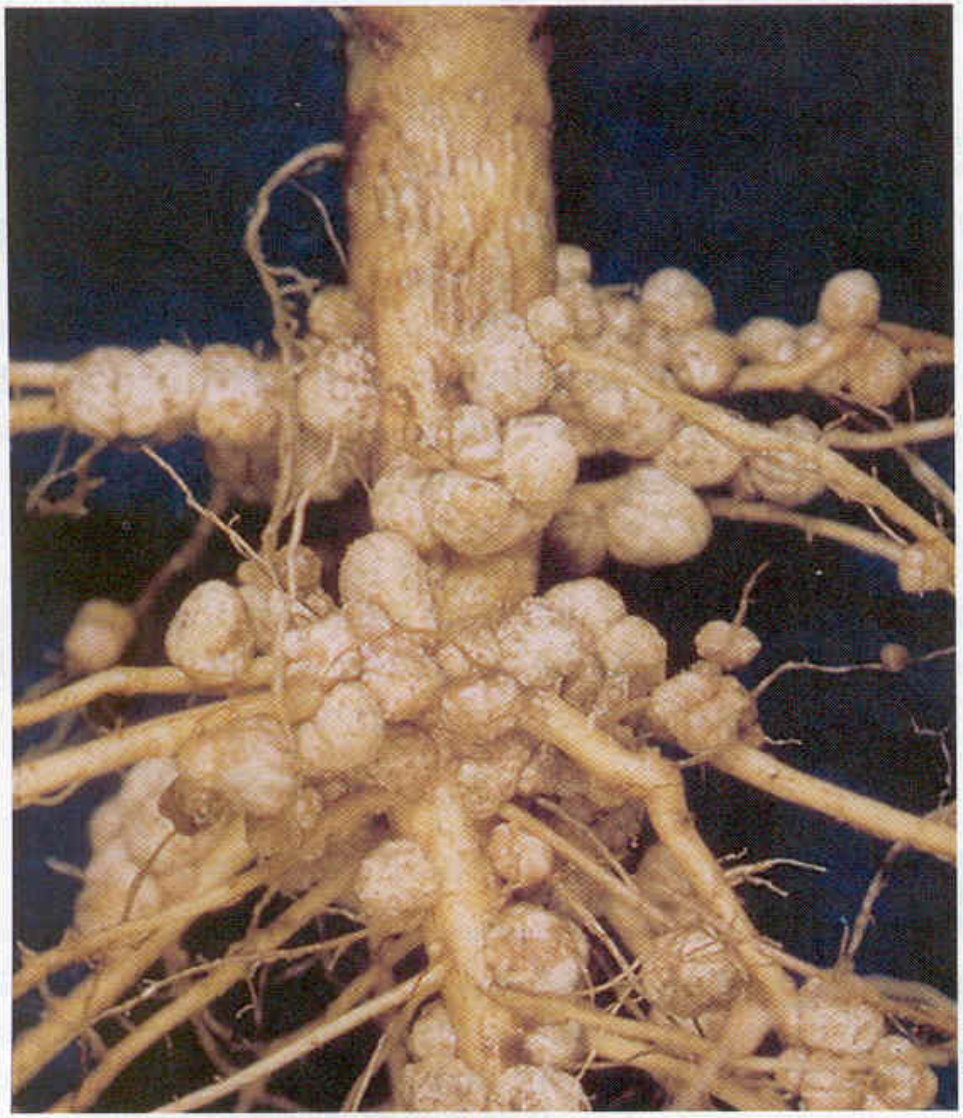
The *Rhizobium*-legume association

Bacterial associations with certain plant families, primarily **legume** species, make the largest single contribution to biological nitrogen fixation in the biosphere

Pea Plant

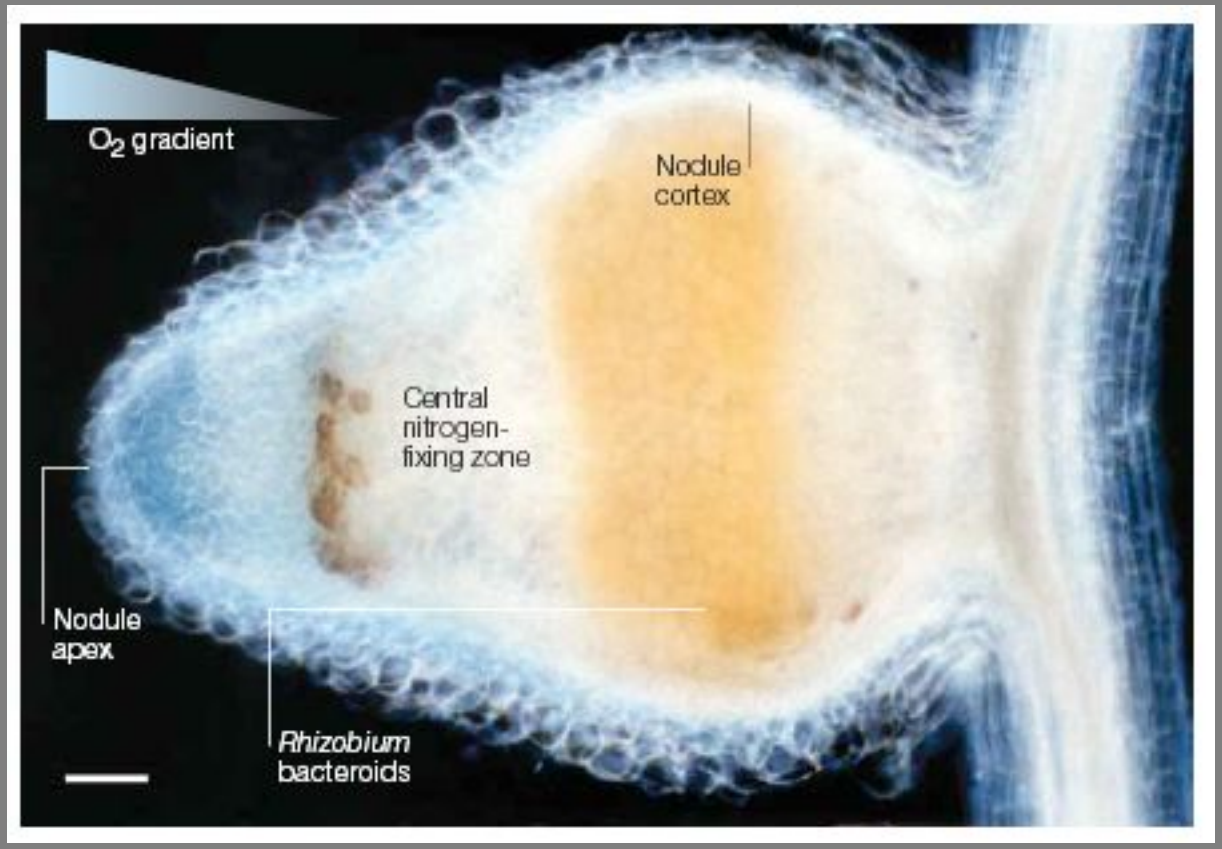
R. leguminosarum nodules

Pink color is leghaemoglobin a protein that carries oxygen to the bacteroids

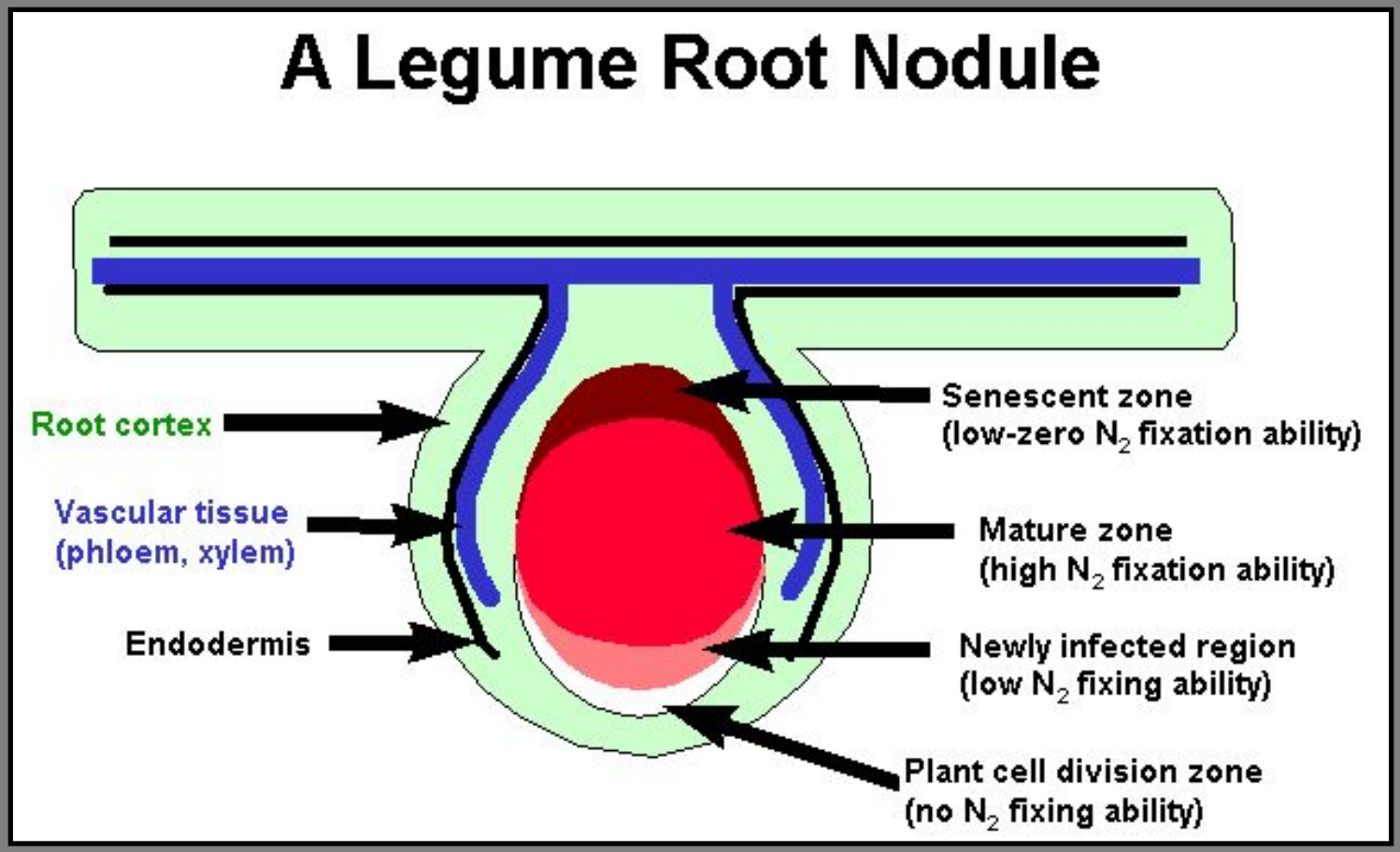


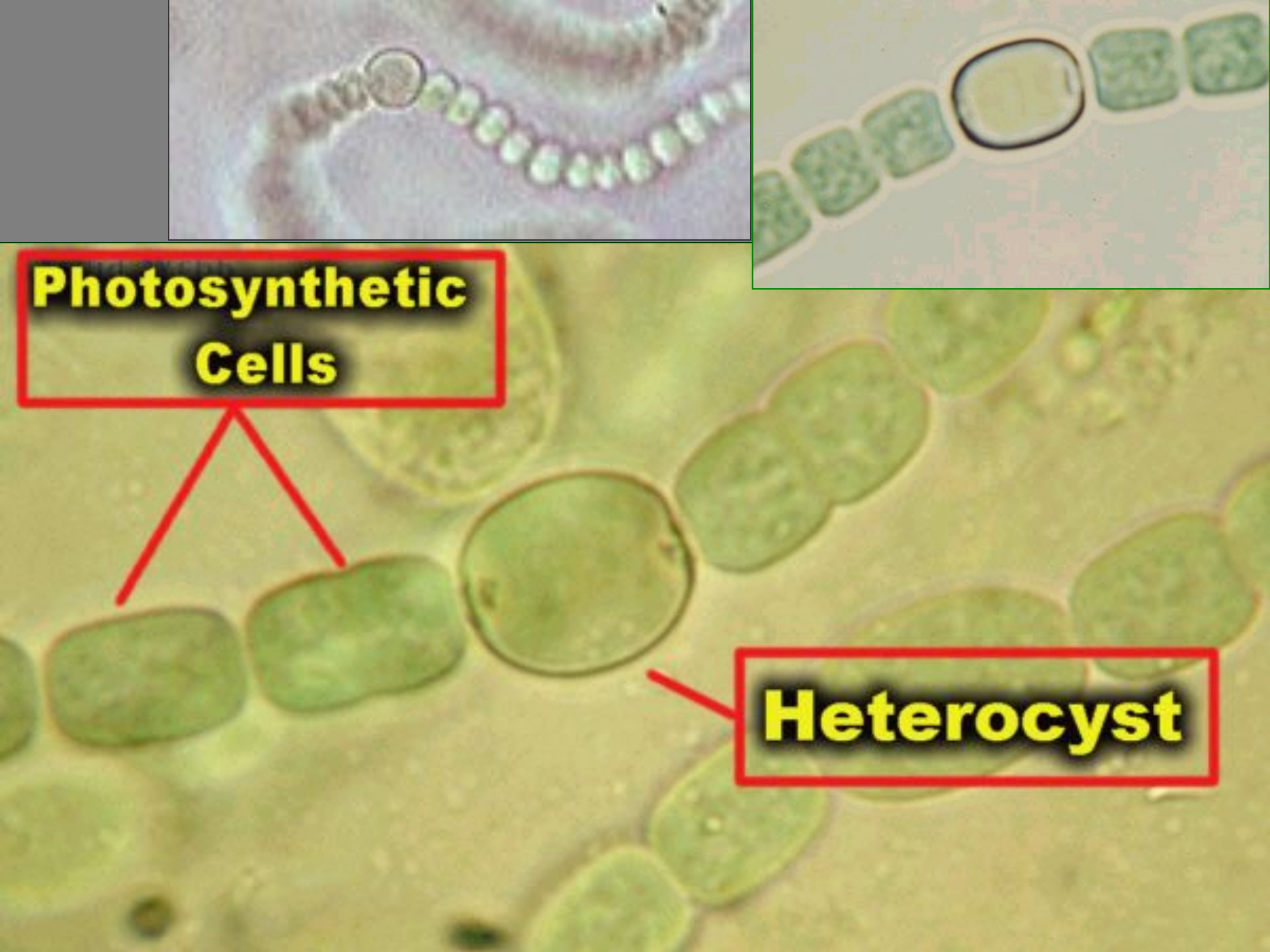
Joe Burton

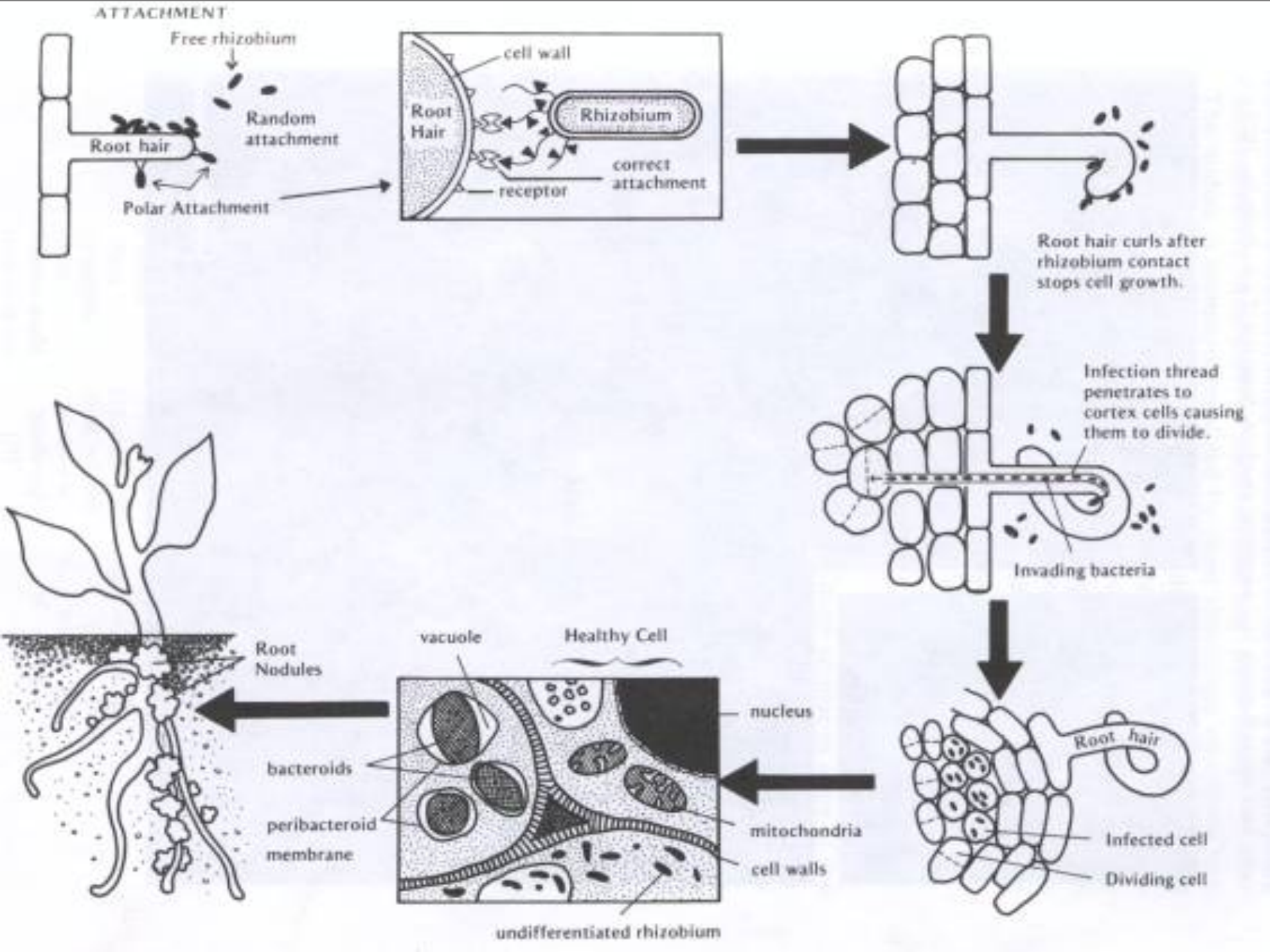
FIGURE 16.73 Soybean root nodules. The nodules develop by infection with *Bradyrhizobium japonicum*.


O_2 gradient
Nodule cortex
Central nitrogen-fixing zone
Nodule apex
Rhizobium bacteroids

Physiology of a legume nodule




Photosynthetic Cells
Heterocyst


ATTACHMENT
Free rhizobium
Random attachment
Root hair
Polar Attachment
cell wall
Root Hair
Rhizobium
correct attachment
receptor
Root hair curls after rhizobium contact stops cell growth.
Infection thread penetrates to cortex cells causing them to divide.
Invading bacteria
Root hair
Infected cell
Dividing cell
nucleus
mitochondria
cell walls
vacuole
Healthy Cell
bacteroids
peribacteroid membrane
undifferentiated rhizobium
Root Nodules

The nodulation process

1. Chemical recognition of root and *Rhizobium*
2. Root hairs curl
3. Formation of infection threads
4. Invasion of the roots by *Rhizobia*
5. Nodule tissue forms
6. Bacteria convert to bacteriods and begin to form nitrogenase enzyme
7. Legume provides *Rhizobia* with carbon. *Rhizobia* provide the legume with fixed N

The Nodulation Process

- Chemical recognition of roots and Rhizobium
- Root hair curling
- Formation of infection thread
- Invasion of roots by Rhizobia
- Cortical cell divisions and formation of nodule tissue
- Bacteria fix nitrogen which is transferred to plant cells in exchange for fixed carbon

Biological NH_3 creation (nitrogen fixation) accounts for an estimated 170×10^9 kg of ammonia every year. Human industrial production amounts to some 80×10^9 kg of ammonia yearly.

The industrial process (Haber-Bosh process) uses an Fe catalyst to dissociate molecules of N_2 to atomic nitrogen on the catalyst surface, followed by reaction with H_2 to form ammonia. This reaction typically runs at ~450º C and 500 atmospheres pressure.

These extreme reaction conditions consume a huge amount of energy each year, considering the scale at which NH_3 is produced industrially.

The Dreams…..

If a way could be found to **mimic nitrogenase catalysis**
(a reaction conducted at 0.78 atmospheres N_2 pressure
and ambient temperatures), huge amounts of energy
(and money) could be saved in industrial ammonia production.

If a way could be found to
transfer the capacity to form N-fixing symbioses
from a typical legume host
to an important non-host crop species such as corn or wheat,
far less fertilizer
would be needed to be produced and applied
in order to sustain crop yields

Because of its current and potential **economic importance**, the interaction between Rhizobia and leguminous plants has been intensively studied.

Our understanding of the process by which these two symbionts establish a functional association is still not complete, but it has provided a **paradigm** for many aspects of cell-to-cell communication between microbes and plants (e.g. during pathogen attack), and even between cells within plants (e.g. developmental signals; fertilization by pollen).

Symbiotic Rhizobia are classified in two groups:

Fast-growing ***Rhizobium*** spp. whose nodulation functions (**nif, fix**) are encoded on their symbiotic megaplasmids (**pSym**)

Slow-growing ***Bradyrhizobium*** spp. whose N-fixation and nodulation functions are encoded on their chromosome.

There are also two types of nodule that can be formed:

determinate

and

indeterminate

This outcome is controlled by the plant host

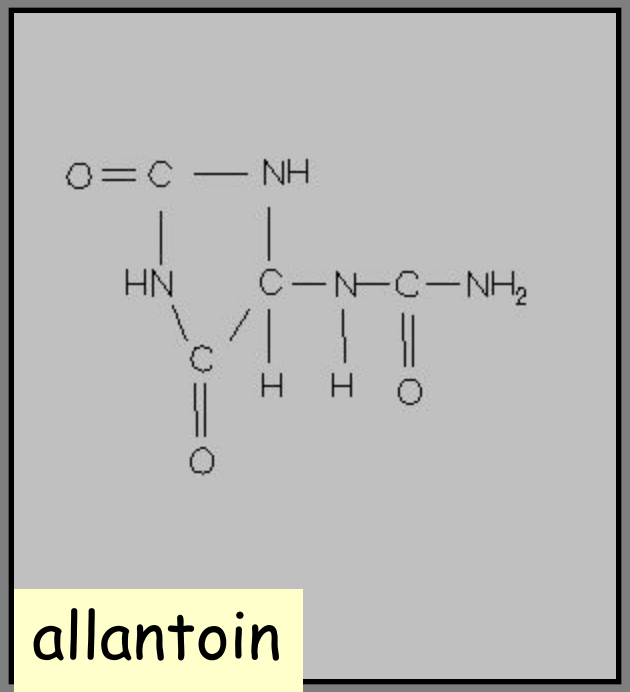
Determinate nodules

Formed on **tropical legumes** by *Rhizobium* and *Bradyrhizobium*

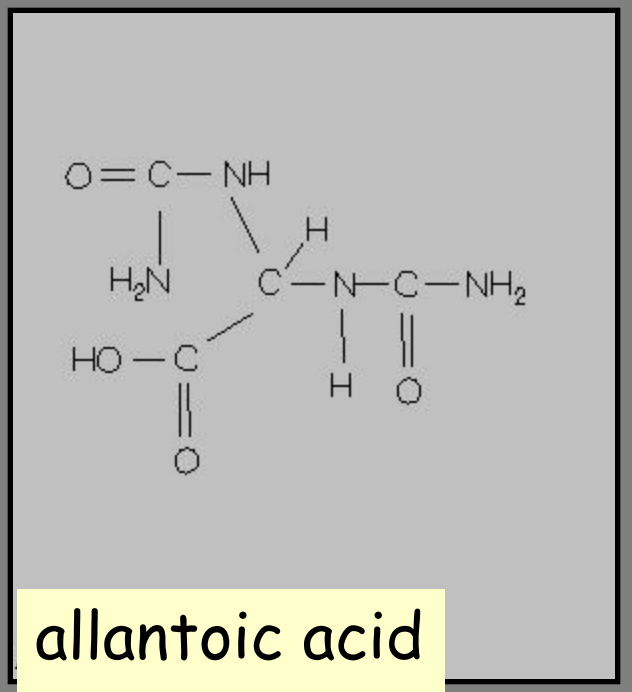
Meristematic activity not persistent - present only during early stage of nodule formation;
after that, cells simply expand rather than divide, to form **globose nodules.**

Nodules arise just below epidermis; largely internal vascular system

Uninfected cells dispersed throughout nodule; equipped to assimilate NH_4^+ as **ureides** (allantoin and allantoic acid)



allantoin



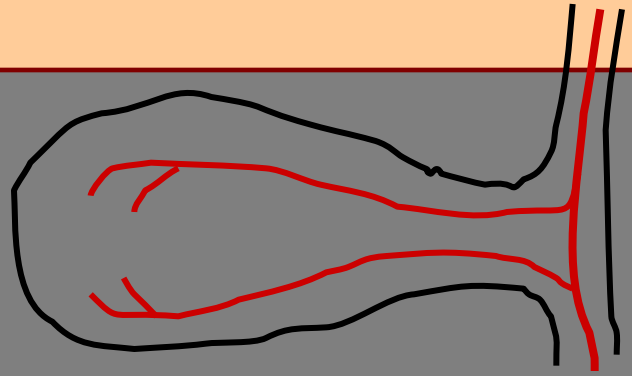
allantoic acid

Indeterminate nodules

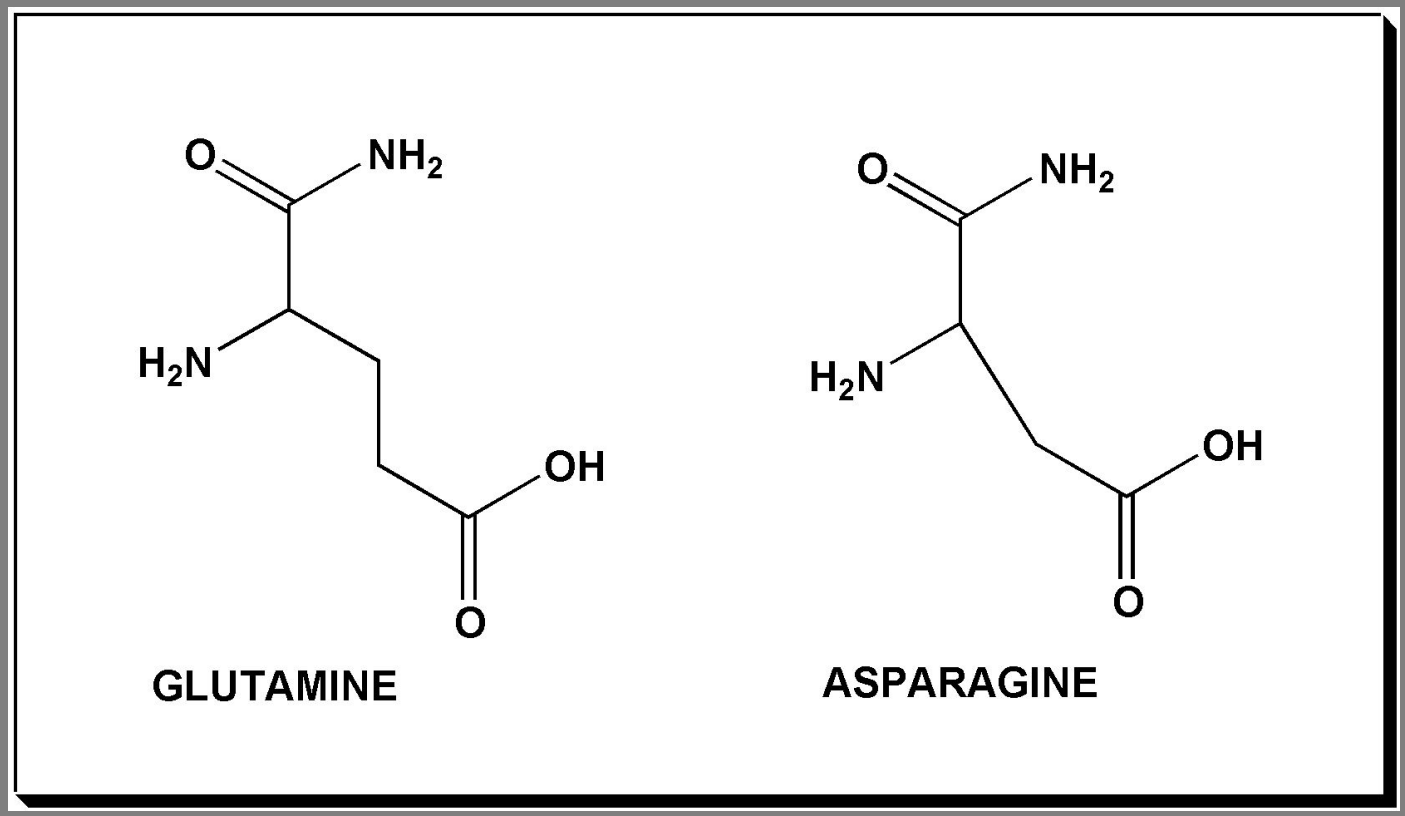
Formed on **temperate** legumes
(pea, clover, alfalfa);
typically by *Rhizobium* spp.

Cylindrical nodules with a **persistent meristem**;
nodule growth creates zones of different developmental stages

Nodule arises near endodermis, and nodule vasculature clearly connected with root vascular system



Uninfected cells of indeterminate nodules assimilate NH_4^+ as **amides** (asparagine, glutamine)



Rhizobium

- establish highly specific symbiotic associations with legumes
  - form **root nodules**
  - fix nitrogen within root nodules
  - nodulation genes are present on large plasmid

Rhizobium-legume symbioses

Host plant	Bacterial symbiont
Alfalfa	*Rhizobium meliloti*
Clover	*Rhizobium trifolii*
Soybean	*Bradyrhizobium japonicum*
Beans	*Rhizobium phaseoli*
Pea	*Rhizobium leguminosarum*
Sesbania	*Azorhizobium caulinodans*

Complete listing can be found at at:
http://cmgm.stanford.edu/~mbarnett/rhiz.htm

Both plant and bacterial factors determine specificity

TABLE 16.8 Major cross-inoculation groups of leguminous plants

Host plant	Nodulated by
Pea	*Rhizobium leguminosarum* biovar *viciae*[a]
Bean	*Rhizobium leguminosarum* biovar *phaseoli*[a]
Bean	*Rhizobium tropici*
Lotus	*Mesorhizobium loti*
Clover	*Rhizobium leguminosarum* biovar *trifolii*[a]
Alfalfa	*Sinorhizobium meliloti*
Soybean	*Bradyrhizobium japonicum*
Soybean	*Bradyrhizobium elkanii*
Soybean	*Rhizobium fredii*
Sesbania rostrata (a tropical legume)	*Azorhizobium caulinodans*

a Several varieties (biovars) of *Rhizobium leguminosarum* exist, each capable of nodulating a different legume.

Typical Associations (cross-inoculation groups)

R.l.* biovar *viciae
colonizes **pea** (*Pisum* spp.) and vetch
(temperate; indeterminate nodules)

R.l.* biovar *trifolii
colonizes **clover** (*Trifolium* spp.)
(temperate; indeterminate nodules)

Rhizobium leguminosarum* biovar *phaseoli
colonizes **bean** (*Phaseolus* spp.)
(tropical; determinate nodules)

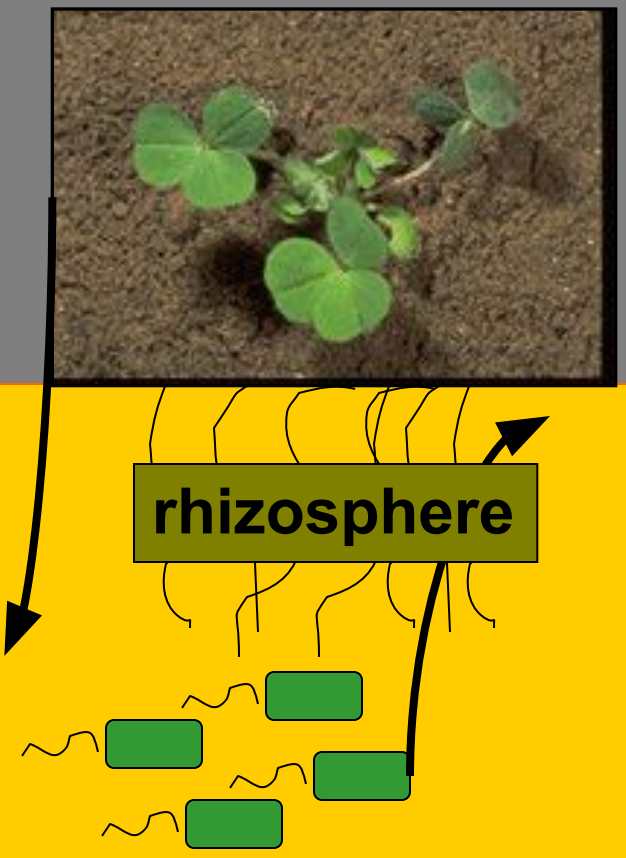
Rhizobium meliloti
colonizes **alfalfa** (*Medicago sativa*)
temperate; indeterminate nodules

Rhizobium fredii
colonizes **soybean** (*Glycine max*)
tropical; determinate nodules

Bradyrhizobium japonicum
colonizes **soybean**
tropical; determinate nodules

Rhizobium NGR 234
colonizes ***Parasponia*** and tropicals;
very broad host range

Very early events in the Rhizobium-legume symbiosis

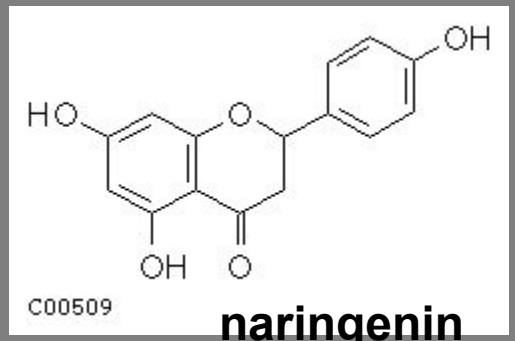


Flavonoids
nod-gene
inducers

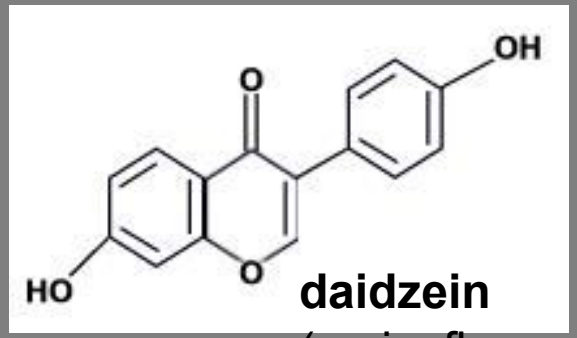
Nod-facto
r

Nodule development process

1. Bacteria encounter root;
they are chemotactically attracted toward specific plant chemicals (**flavonoids**) exuding from root tissue, especially in response to nitrogen limitation

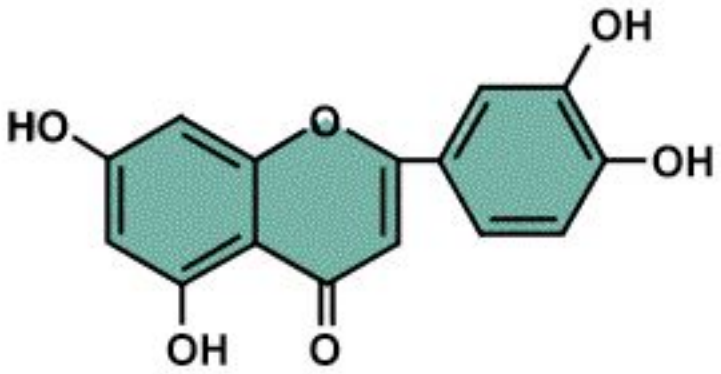


naringenin
(a flavanone)



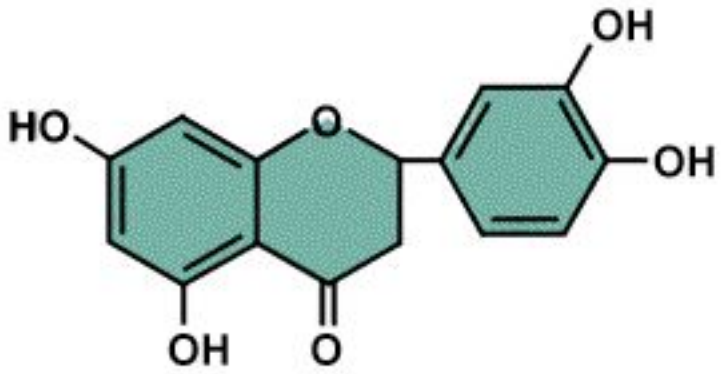
daidzein
(an isoflavone)

Inducers of nodulation in *Rhizobium leguminosarum bv viciae*



5, 7, 3', 4'-Tetrahydroxyflavone

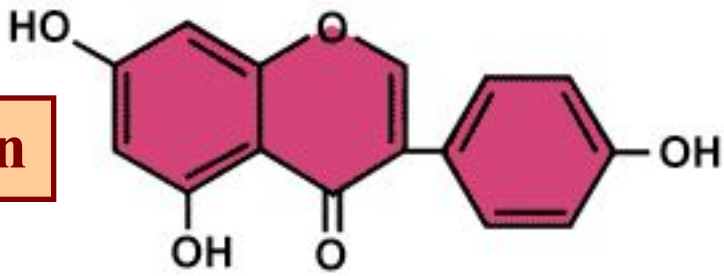
luteolin



5, 7, 3', 4'-Tetrahydroxyflavone

eriodictyol

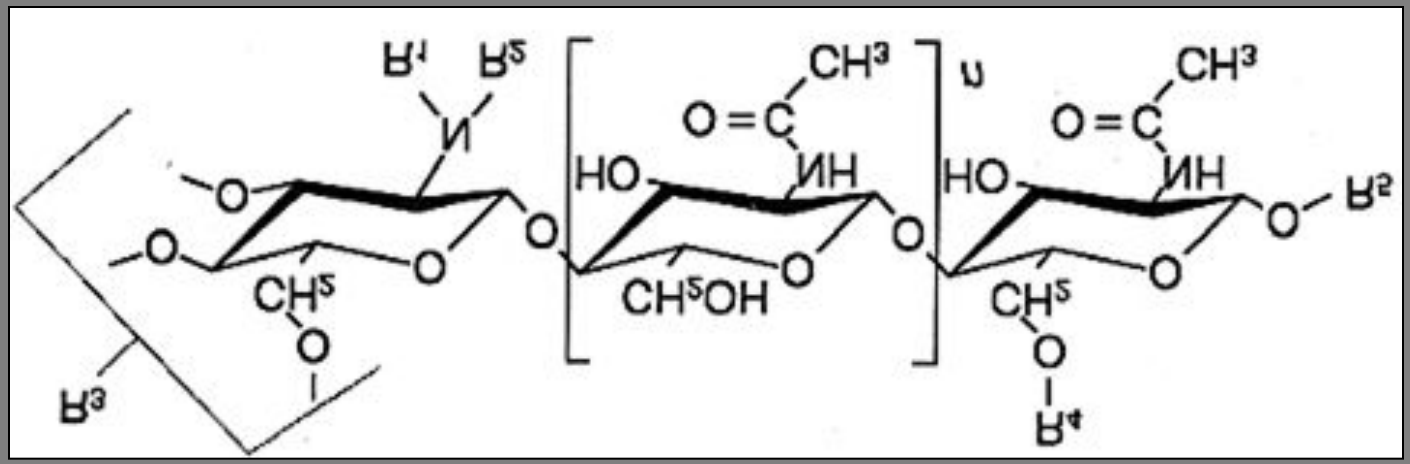
Inhibitor of nodulation



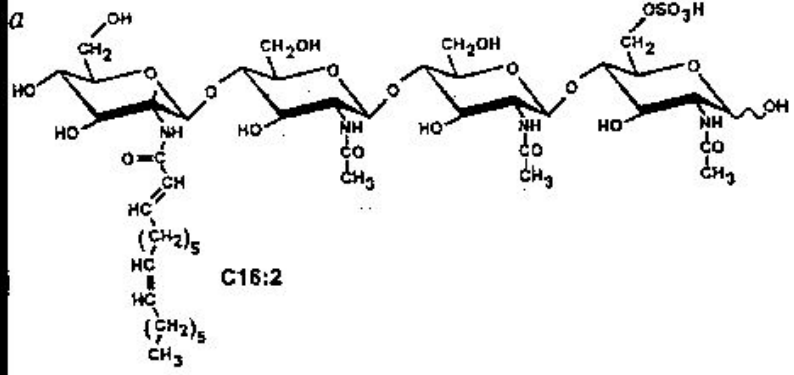
5, 7, 4'-Trihydroxyisolflavone

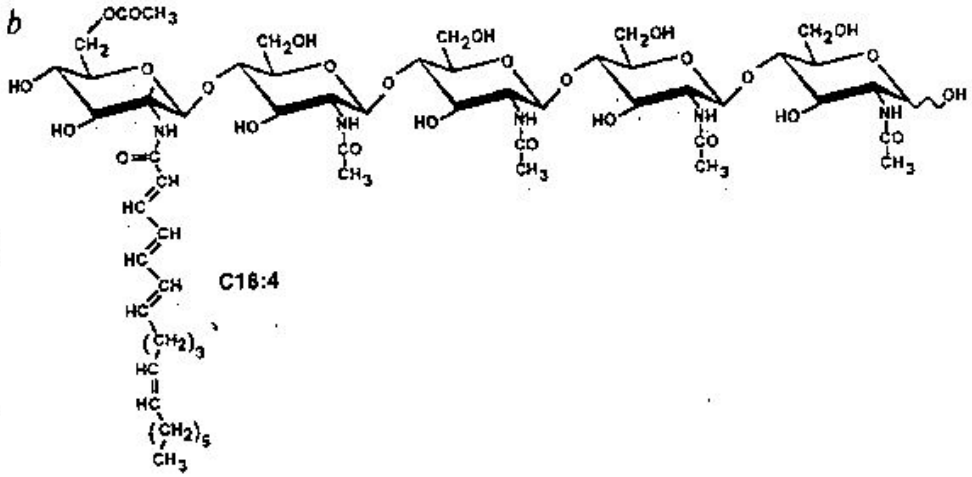
genistein

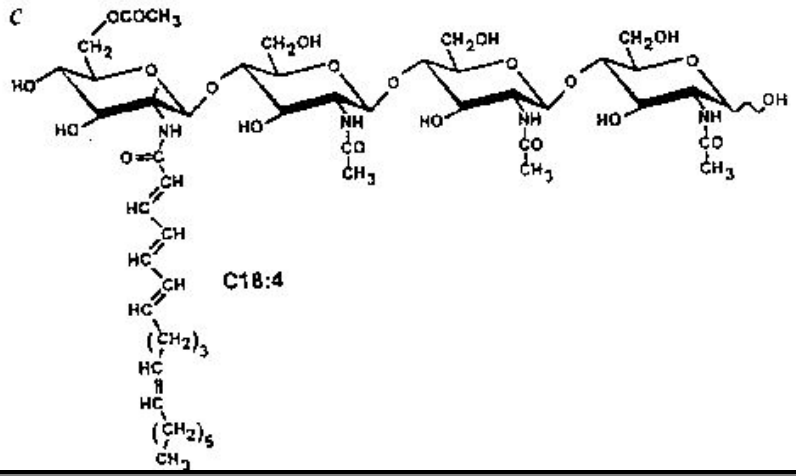
2. Bacteria attracted to the root attach themselves to the root hair surface and secrete specific **oligosaccharide** signal molecules (**nod factors**).



nod factor





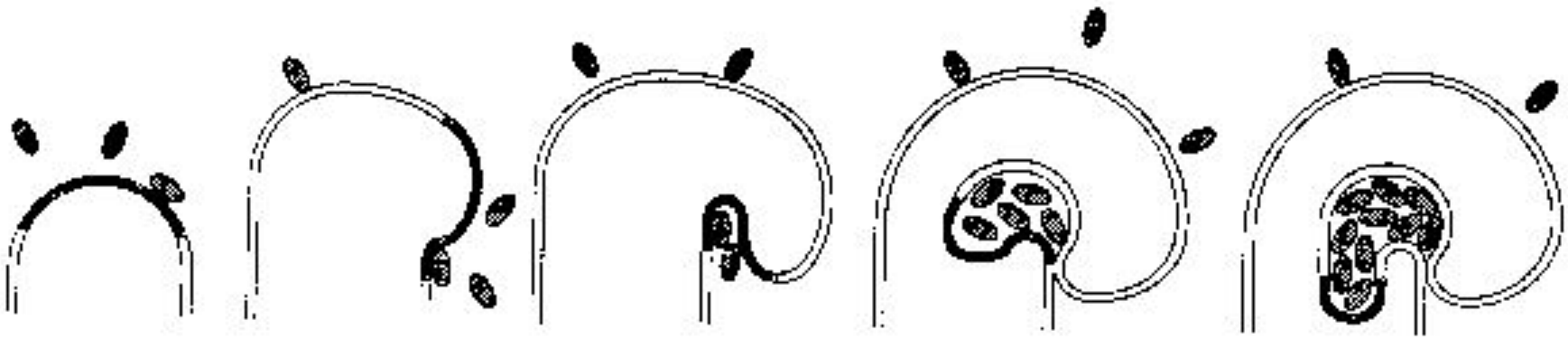


Examples of different nod factors

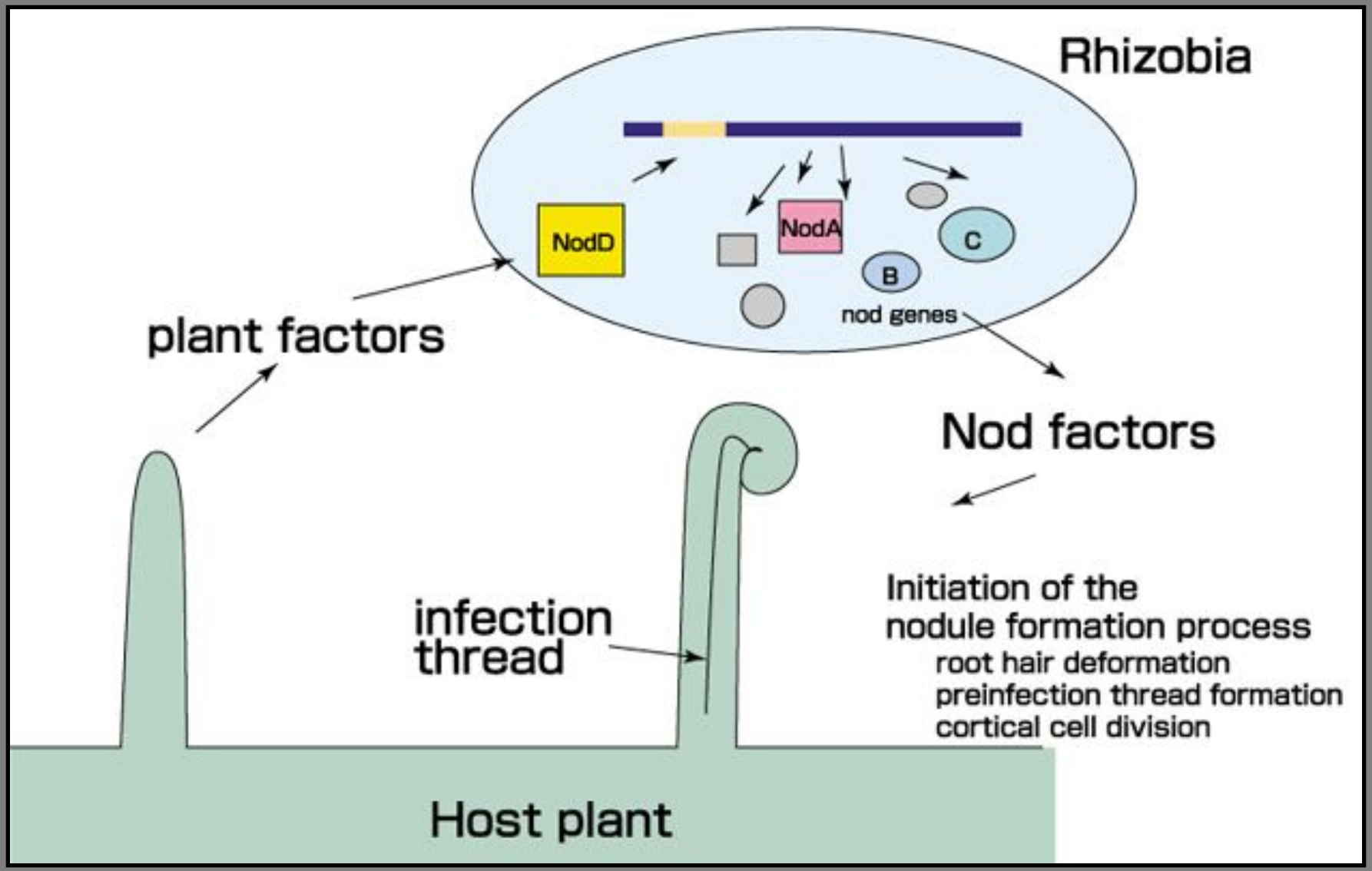
3. In response to oligosaccharide signals, the root hair becomes deformed and **curls** at the tip; bacteria become enclosed in small pocket.

Cortical **cell division** is induced within the root.

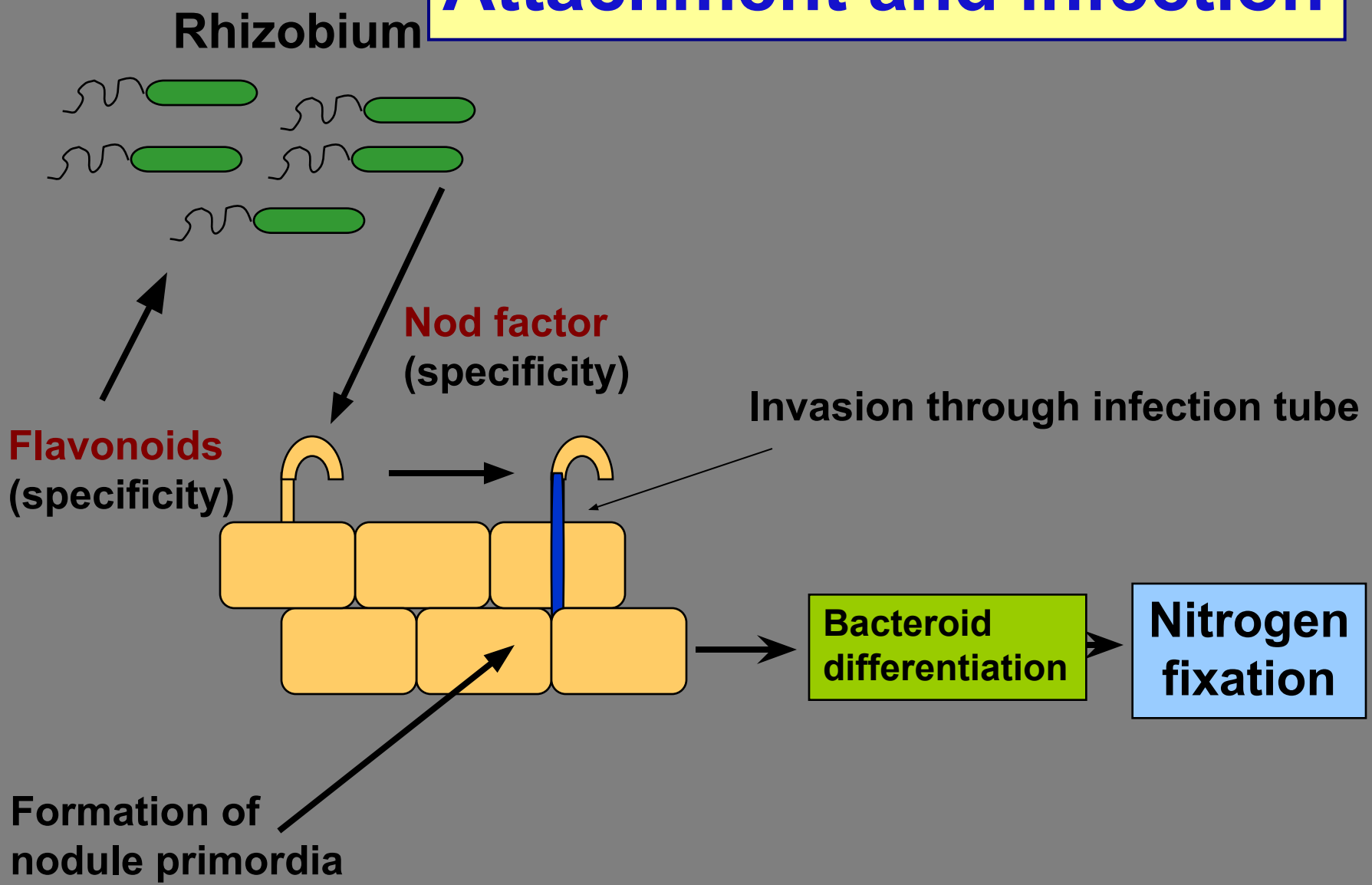
Root hair attachment and curling

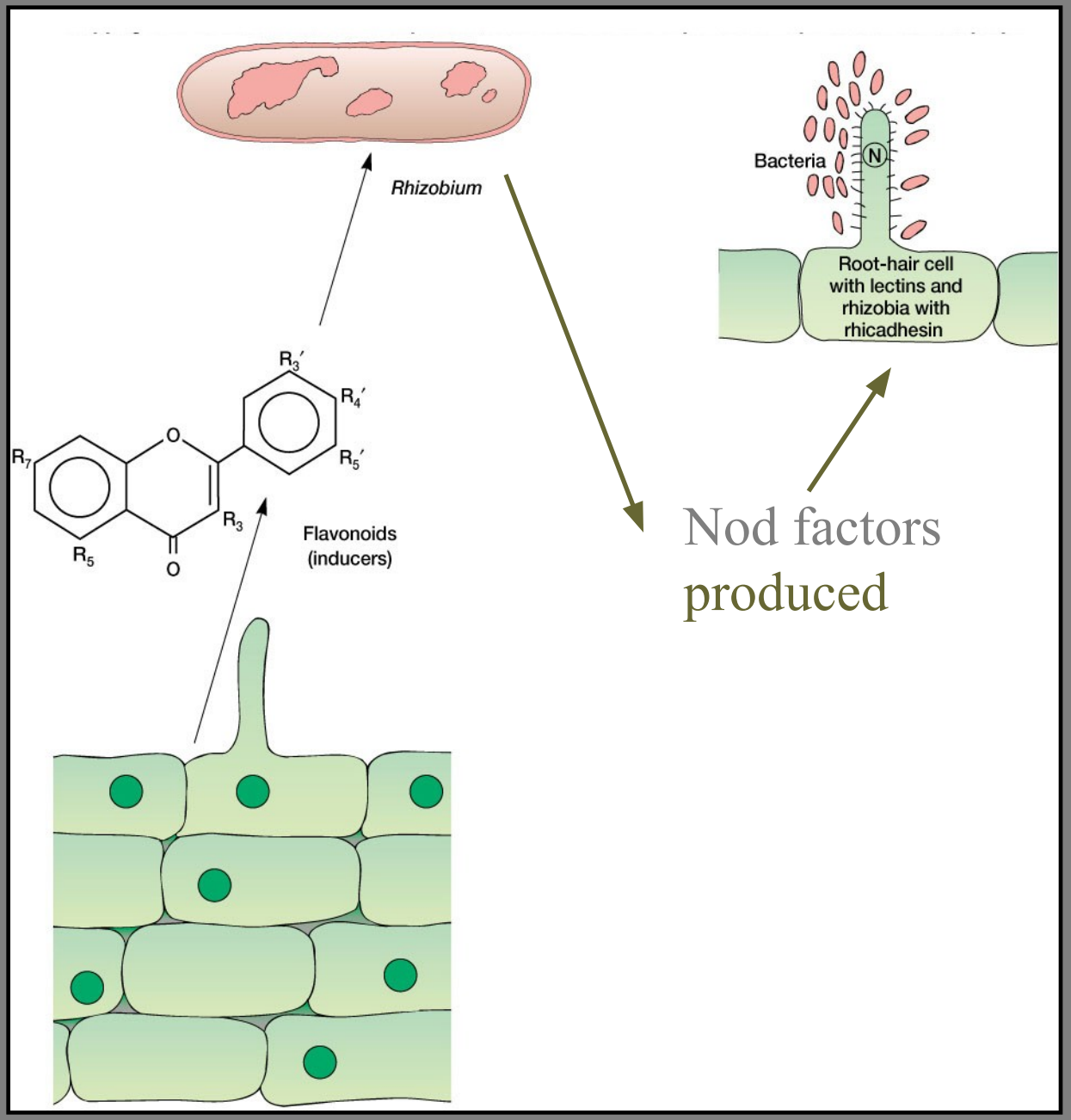


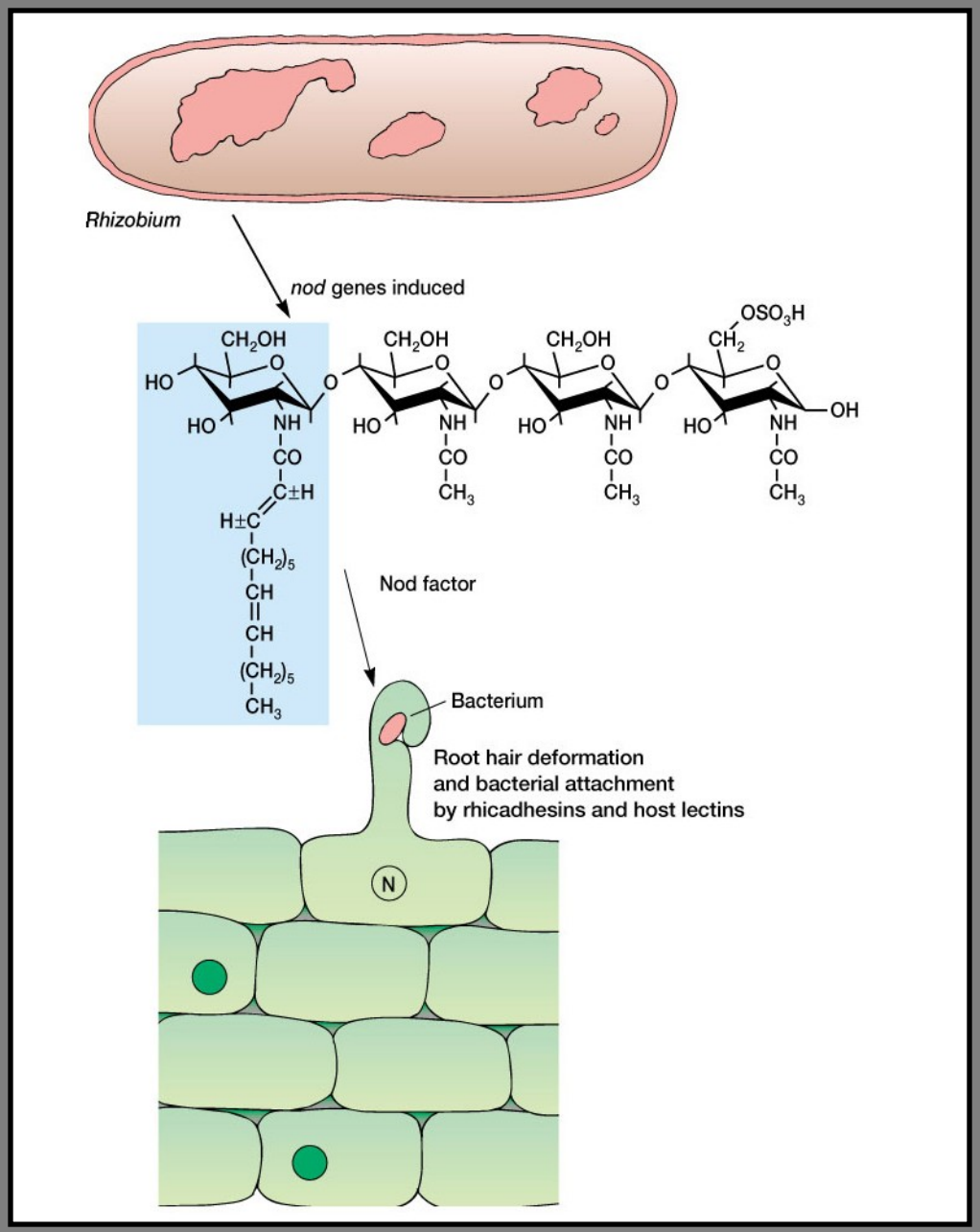

root hair beginning to curl
Rhizobium cells


Rhizobia
NodD
NodA
C
B
nod genes
plant factors
Nod factors
infection
thread
Initiation of the
nodule formation process
root hair deformation
preinfection thread formation
cortical cell division
Host plant

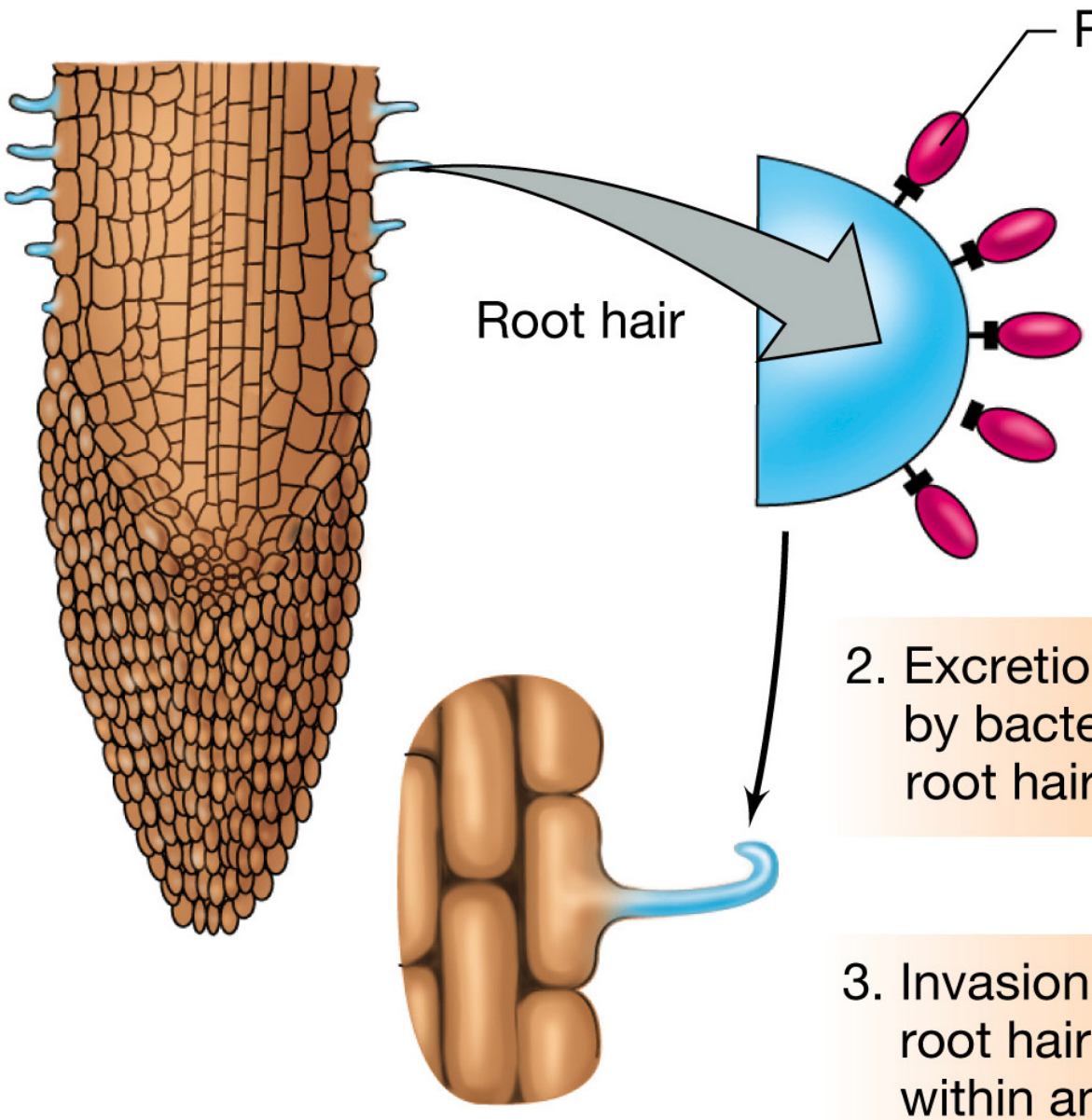
Attachment and infection




Rhizobium
Bacteria
N
Root-hair cell with lectins and rhizobia with rhicadhesin
Flavonoids (inducers)
Nod factors produced

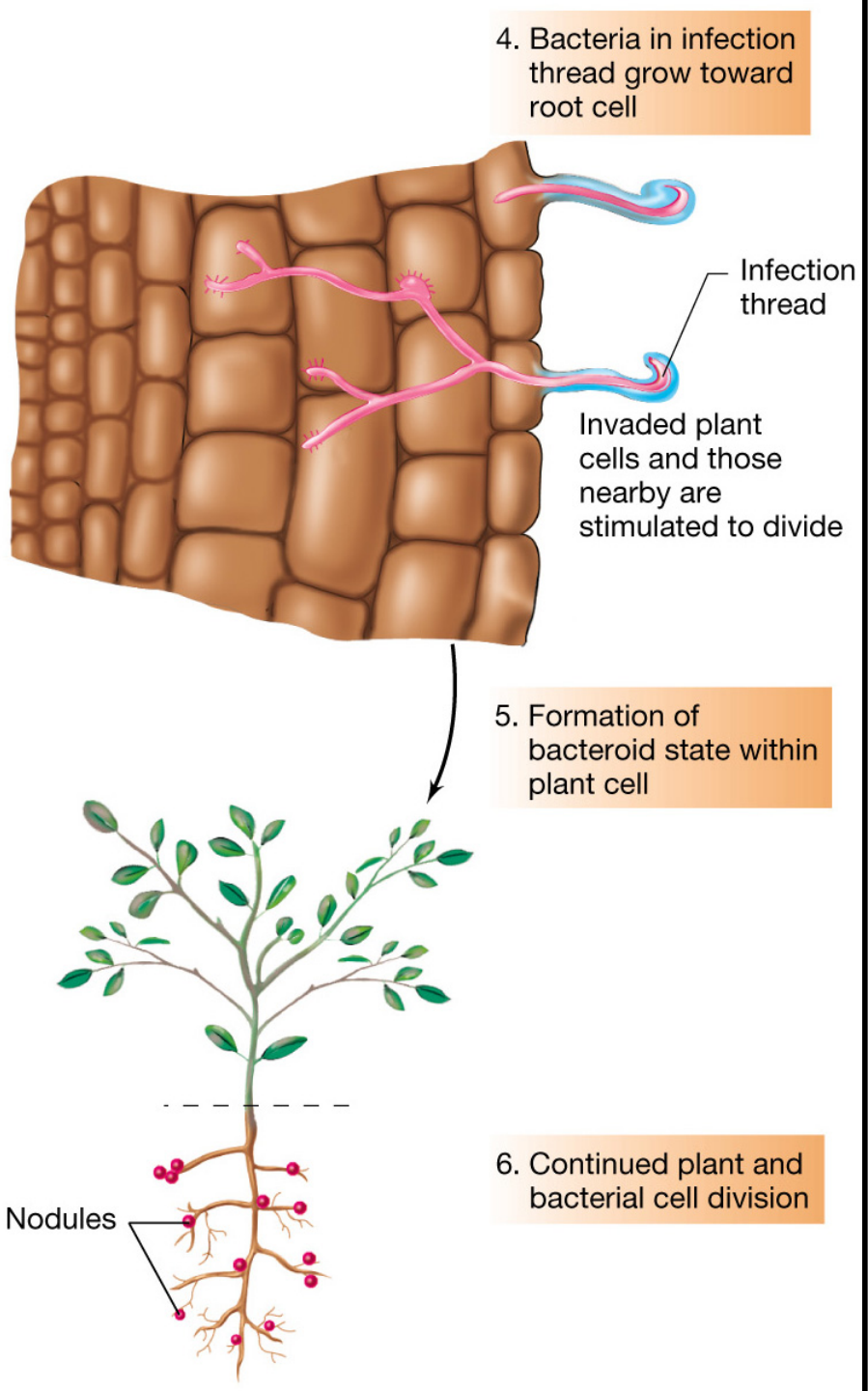

Rhizobium
nod genes induced
Nod factor
Bacterium
Root hair deformation
and bacterial attachment
by rhicadhesins and host lectins
N

1. Recognition and attachment (rhicadhesin-mediated)

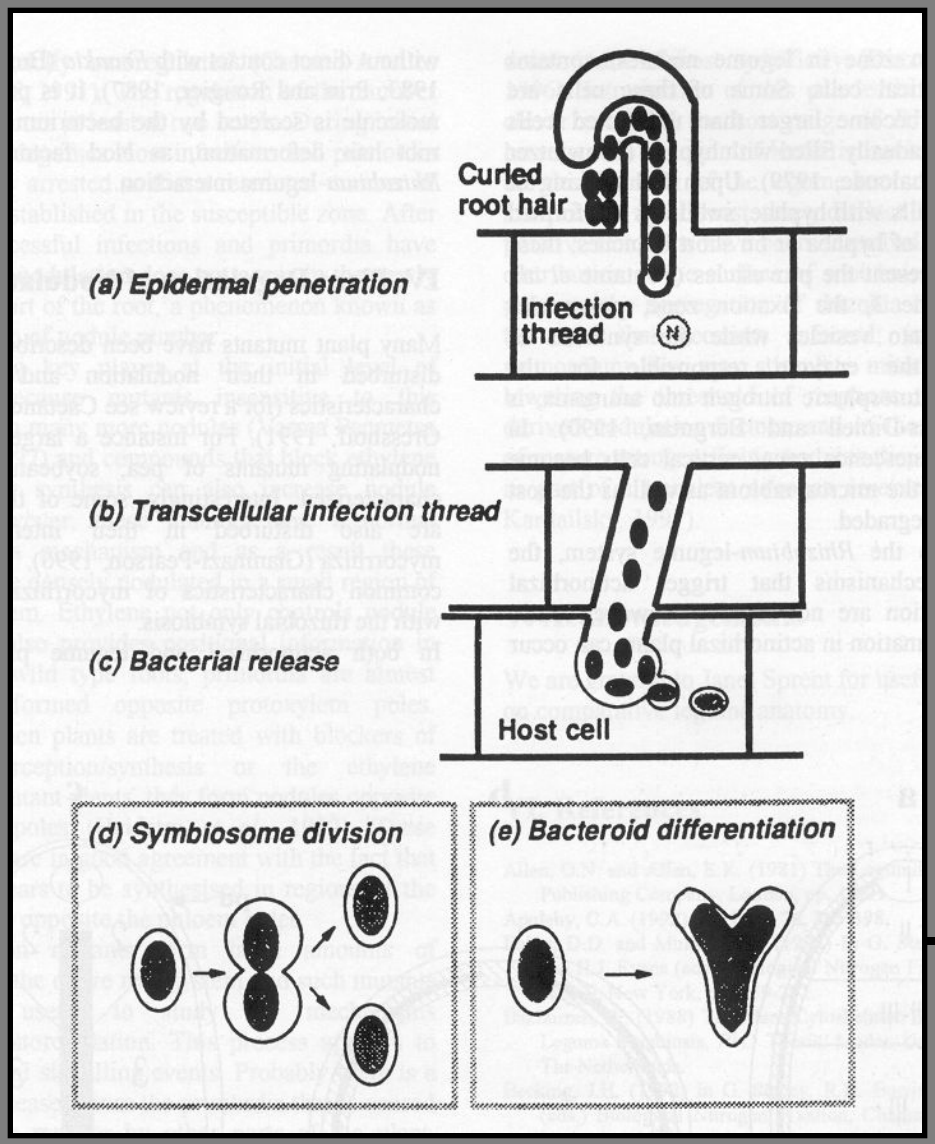


2. Excretion of nod factors by bacterium causing root hair curling

3. Invasion. Rhizobia penetrate root hair and multiply within an "infection thread"


4. Bacteria in infection thread grow toward root cell
Infection thread
Invaded plant cells and those nearby are stimulated to divide
5. Formation of bacteroid state within plant cell
Nodules
6. Continued plant and bacterial cell division

Nodule development



Enlargement of the nodule, nitrogen fixation and exchange of nutrients

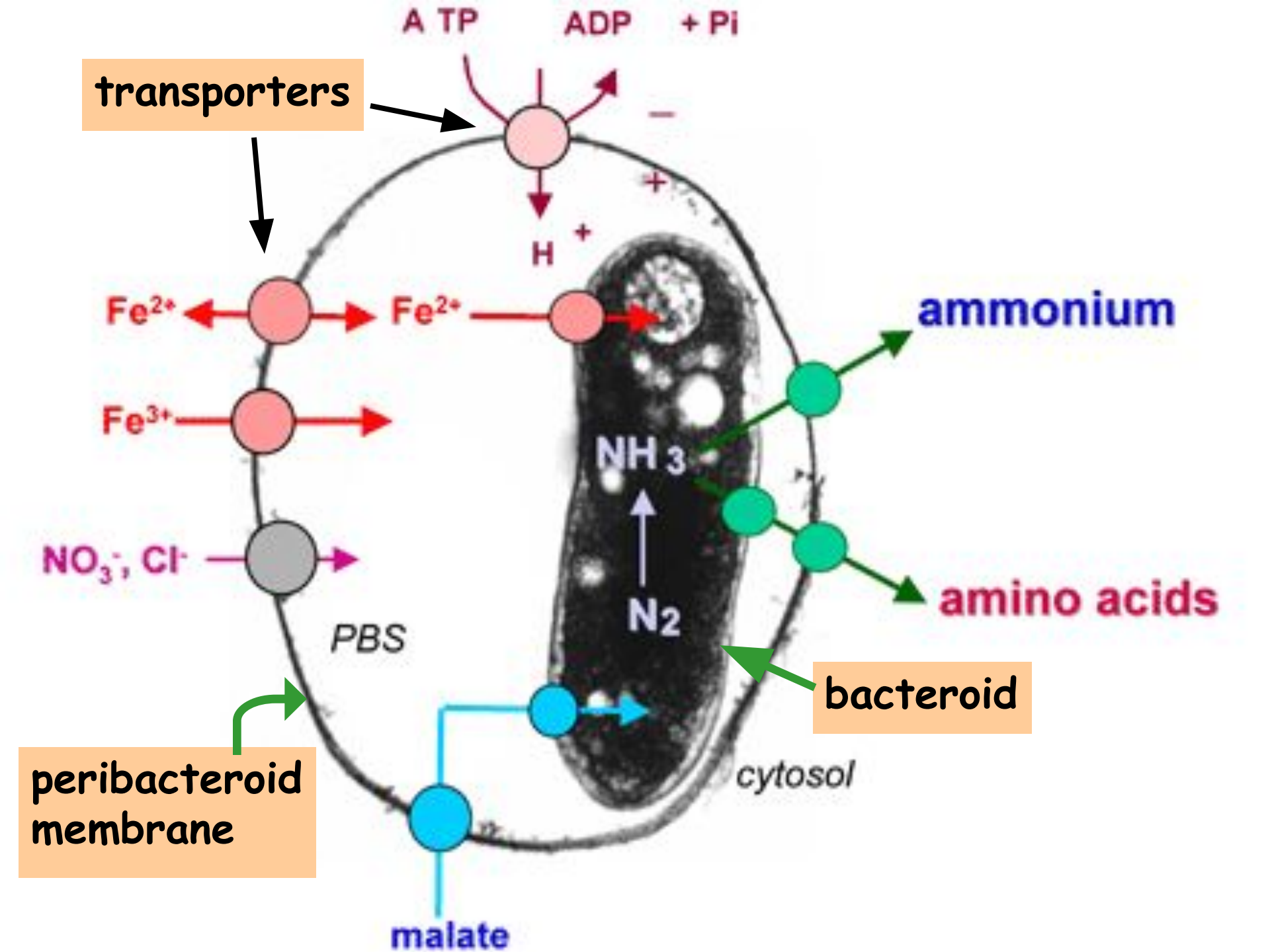
5. Infection thread penetrates through several layers of cortical cells and then ramifies within the cortex. Cells in advance of the thread divide and organize themselves into a nodule primordium.

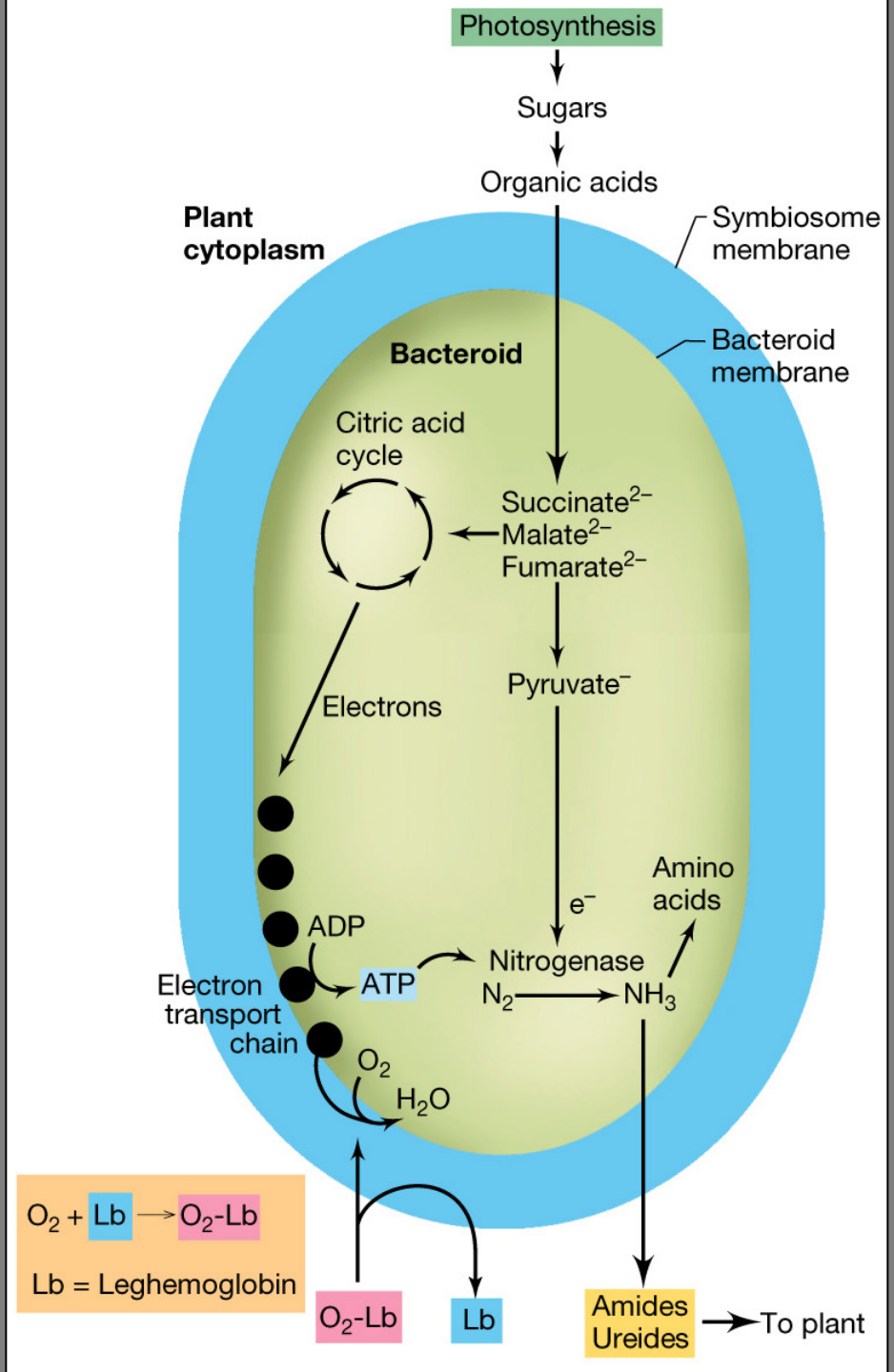
6. The branched infection thread enters the nodule primordium zone and penetrates individual primordium cells.

7. Bacteria are released from the infection thread into the cytoplasm of the host cells, but remain surrounded by the **peribacteroid membrane**. Failure to form the PBM results in the activation of host defenses and/or the formation of ineffective nodules.

8. Infected root cells swell and cease dividing. Bacteria within the swollen cells change form to become endosymbiotic **bacteroids,** which begin to fix nitrogen.

The nodule provides an **oxygen-controlled** environment (leghemoglobin = pink nodule interior) structured to facilitate transport of reduced nitrogen metabolites from the bacteroids to the plant vascular system, and of photosynthate from the host plant to the bacteroids.


A TP
ADP
+ Pi
transporters
H +
Fe2+
Fe2+
Fe3+
NO3-, Cl-
PBS
NH 3
N2
ammonium
amino acids
bacteroid
cytosol
peribacteroid membrane
malate


Photosynthesis
Sugars
Organic acids
Plant cytoplasm
Symbiosome membrane
Bacteroid
Bacteroid membrane
Citric acid cycle
Succinate2–
Malate2–
Fumarate2–
Pyruvate–
Electrons
e–
Amino acids
ADP
ATP
Nitrogenase
N2
NH3
Electron transport chain
O2
H2O
O2 + Lb → O2-Lb
Lb = Leghemoglobin
O2-Lb
Lb
Amides
Ureides
To plant

Types of bacterial functions involved in nodulation and nitrogen fixation

nod (nodulation) and nol (nod locus) genes

mutations in these genes block nodule formation or alter host range

most have been identified by transposon mutagenesis, DNA sequencing and protein analysis, in *R. meliloti*, *R. leguminosarum* bv *viciae* and *trifolii*

fall into four classes:
- nodD
- nodA, B and C (common nodgenes)
- hsn (host-specific nod genes)
- other nod genes

Gene clusters on *R. meliloti* pSym plasmid

(nol) (nod) (nif) (fix)

F G H I N D_1 A B C I J Q P G E F H D_3 E K D H A B C

N M L R E F D A B C I J T C B A H D K E N

Gene clusters on *R. leguminosarum* bv *trifolii* pSym plasmid

--- D_2 D_1 Y A B C S U I J ---

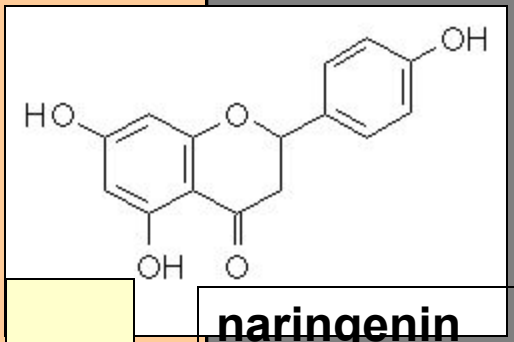
Gene cluster on *Bradyrhizobium japonicum* chromosome

Nod D (the sensor)

the **nod D** gene product recognizes molecules (phenylpropanoid-derived **flavonoids**) produced by plant roots and becomes activated as a result of that binding

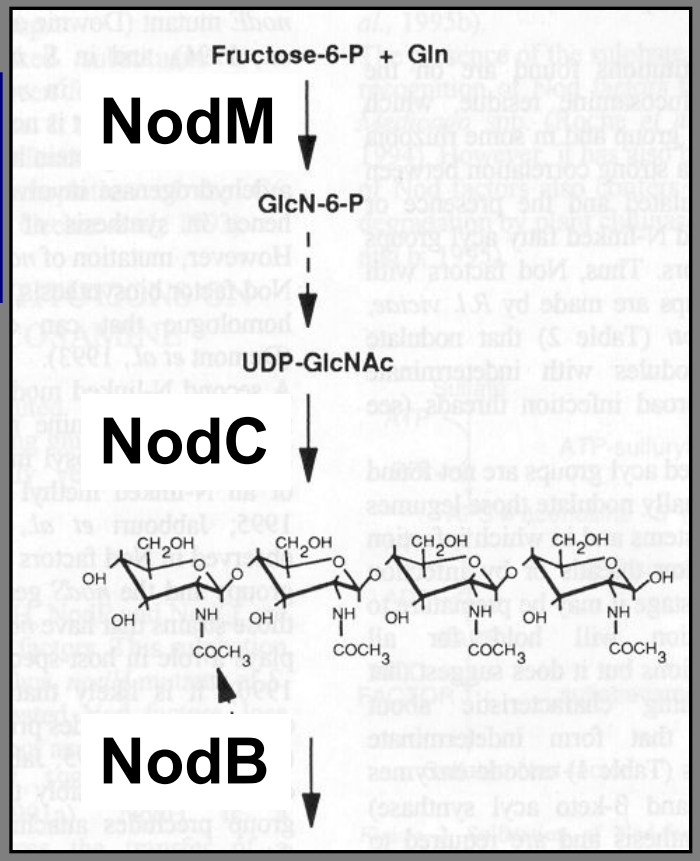
activated nodD protein positively controls the expression of the other genes in the nod gene "regulon" (signal transduction)

different nodD alleles recognize **various flavonoid** structures with different affinities, and respond with differential patterns of nod gene activation

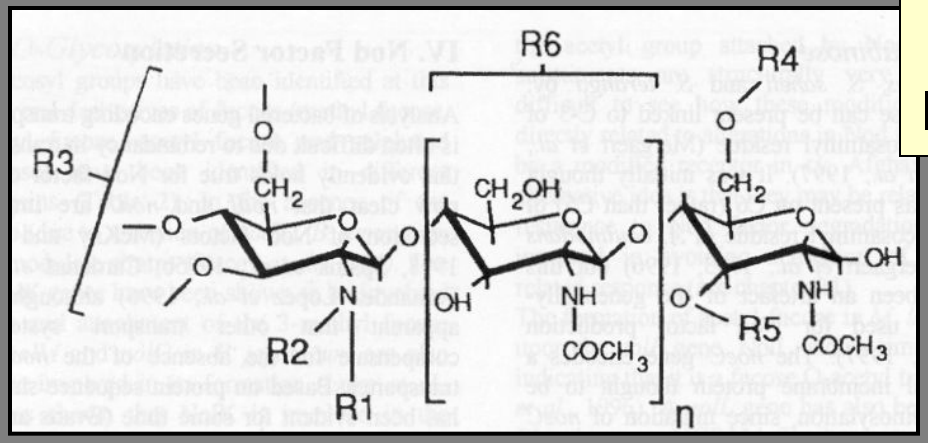


naringenin
(**a flavanone**)

Nod factor biosynthesis



Nod factor R-group "decorations" determine host specificity



Nod Factor: a lipooligosaccharide

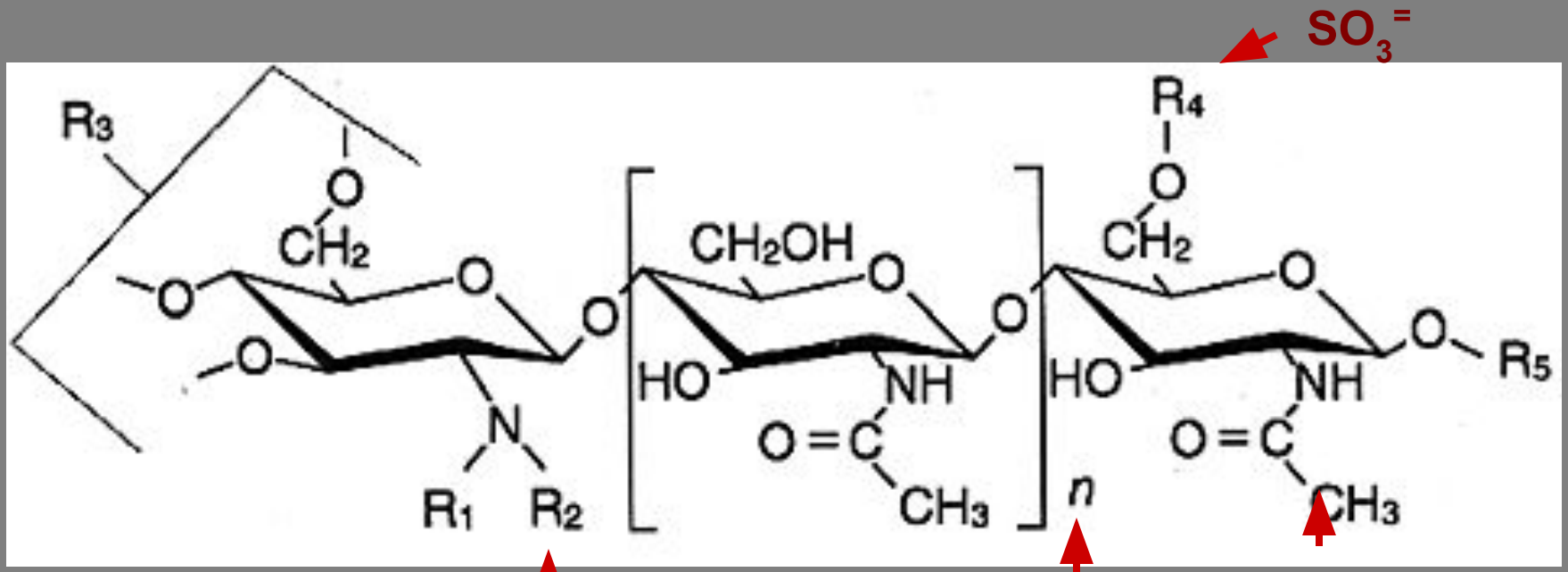
Common nod genes - nod ABC

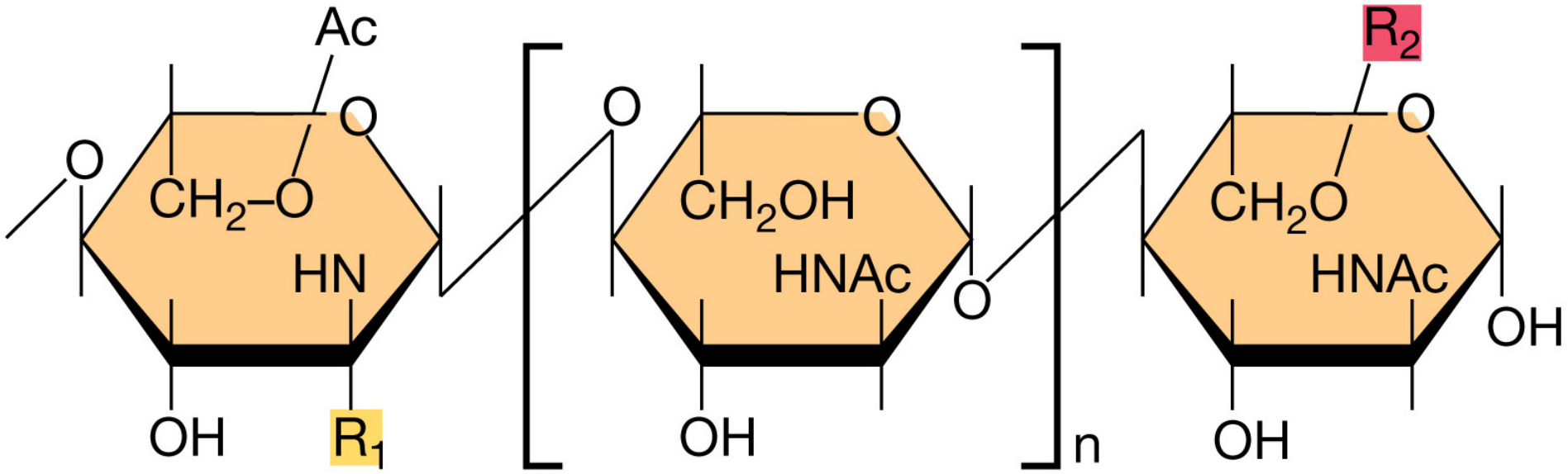
mutations in nodA,B or C completely abolish the ability of the bacteria to nodulate the host plant; they are found as part of the nod gene "regulon" in <u>all</u> Rhizobia (∴ common)

products of these genes **are required** for bacterial induction of root cell hair deformation and root cortical cell division

The **nod ABC** gene products are enzymes responsible for synthesis of diffusible nod factors, whcih are sulfated and acylated *beta*-1,4-oligosaccharides of glucosamine

(other gene products, e.g. NodH, may also be needed for special modifications)





(a)

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

(b)

nod factors are active on host plants at very low concentrations (10^{-8} to 10^{-11} M) but have no effect on non-host species

Host-specific nod genes

mutations in these genes elicit abnormal root reactions on their usual hosts, and sometimes elicit root hair deformation reactions on plants that are not usually hosts

Example:
loss of nodH function in *R. meliloti* results in synthesis of a nod factor that is no longer effective on alfalfa but has gained activity on vetch

The ΔnodH nod factor is now more hydrophobic than the normal factor - no sulfate group on the oligosaccharide.

The role of the nodH gene product is therefore to add a specific sulfate group, and thereby change host specificity

Other nod genes

May be involved in the attachment of the bacteria to the plant surface, or in export of signal molecules, or proteins needed for a successful symbiotic relationship

exo (exopolysaccharide) genes

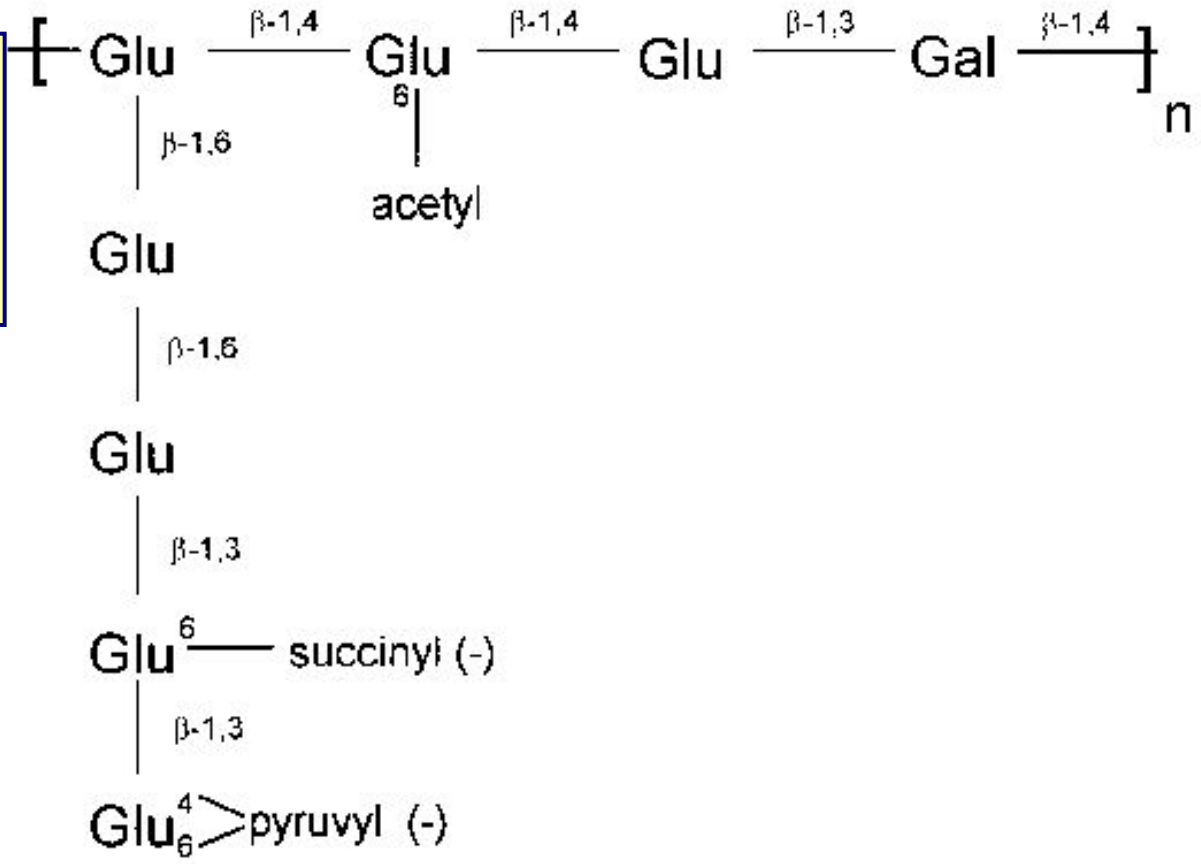
Encode proteins needed for exopolysaccharide synthesis and secretion

In *Rhizobium*-legume interactions that lead to indeterminate nodules, exo mutants cannot invade the plant properly. However, they do provoke the typical plant cell division pattern and root deformation, and can even lead to nodule formation, although these are often empty (no bacteroids).
In interactions that usually produce **determinate** nodules, exo mutations tend to have no effect on the process.

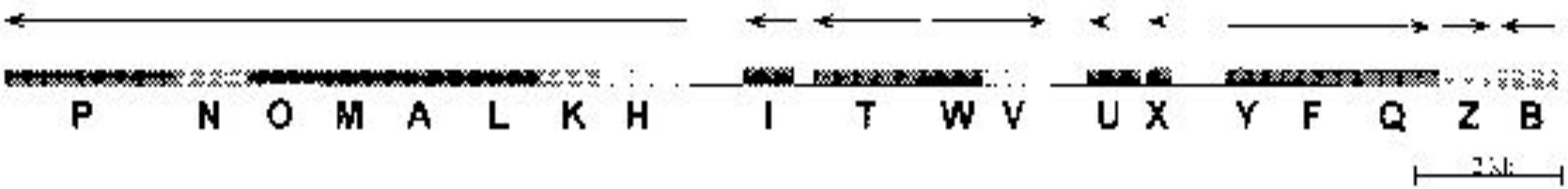
Exopolysaccharides may provide substrate for signal production, osmotic matrix needed during invasion, and/or a recognition or masking function during invasion

example of Rhizobial exopolysaccharide

Succinoglycan



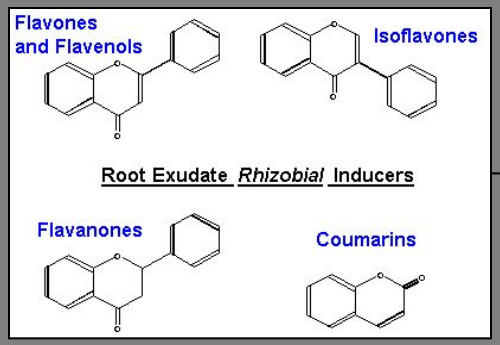
Map of the *exo* Gene Cluster



Functions of the *exo* gene products

- addition of first galactose to lipid carrier
- glucosyltransferase
- octamer modification
- nucleotide sugar biosynthesis
- polymerization or transport
- glycanase
- putative regulatory protein
- function unknown

Sinorhizobium meliloti



nod-gene inducers from alfalfa roots (specificity)

NodD

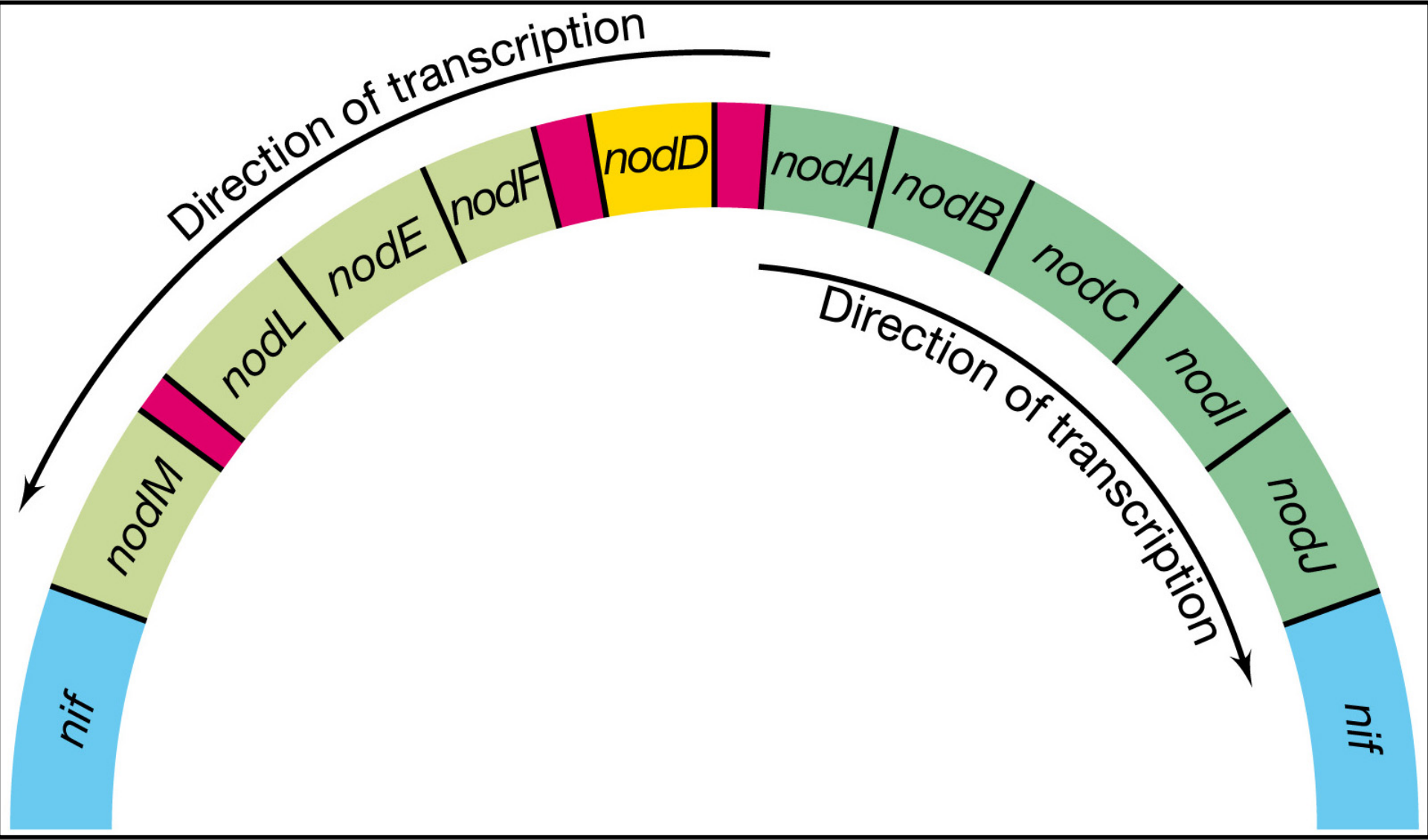
chromosome

plasmid

pSym

activated NodD positively regulates nod genes

nod genes


Direction of transcription
nodM
nodL
nodE
nodF
nodD
nodA
nodB
nodC
nodI
nodJ
nif
nif
Direction of transcription

nif (nitrogen fixation) genes

Gene products are required for symbiotic nitrogen fixation, and for nitrogen fixation in free-living N-fixing species

Example: subunits of nitrogenase

Table 1. The *nif-gene* Products and Their Role (Known or Proposed) in Nitrogen Fixation

nif-GENE	IDENTITY/ROLE
nifH	Dinitrogenase reductase. Obligate electron donor to dinitrogenase during nitrogenase turnover. Also is required for FeMo-co biosynthesis and apodinitrogenase maturation
nifD	α subunit of dinitrogenase. Forms an $a_2ß_2$ tetramer with ß subunit. FeMo-co, the site of substrate reduction, is present buried within the α subunit of dinitrogenase
nifK	ß subunit of dinitrogenase. P-clusters are present at the ß subunit-interface
nifT	Unknown
nifY	In *K. pneumoniae,* aids in the insertion of FeMo-co into apodinitrogenase
nifE	Forms $a_2ß_2$ tetramer with NifN. Required for FeMo-co synthesis. Proposed to function as a scaffold on which FeMo-co is synthesized
nifN	Required for FeMo-co synthesis
nifX	Involved in FeMo-co synthesis. Specific role is not known
nifU	Involved in mobilization of Fe for Fe-S cluster synthesis and repair
nifS	Involved in mobilization of S for Fe-S cluster synthesis and repair
nifV	Homocitrate synthase, involved in FeMo-co synthesis
nifW	Involved in stability of dinitrogenase. Proposed to protect dinitrogenase from 0_2 inactivation
nifZ	Unknown
nifM	Required for the maturation of NifH
nifF	Flavodoxin. Physiologic electron donor to NifH
nifL	Negative regulatory element
nifA	Positive regulatory element
nifB	Required for FeMo-co synthesis. Metabolic product, NifB-co is the specific Fe and S donor to FeMo-co
fdxN	Ferredoxin. In *R. capsulatus,* serves as electron donor to nitrogenase
nifQ	Involved in FeMo-co synthesis. Proposed to function in early MoO_4^{2-} processing
nifJ	Pyruvate:flavodoxin (ferredoxin) oxidoreductase. Involved in electron transport to nitrogenase

fix (fixation) genes

Gene products required to successfully establish a functional N-fixing nodule.
No **fix** homologues have been identified in free-living N-fixing bacteria.

Example: regulatory proteins that monitor and control oxygen levels within the bacteroids

FixL senses the oxygen level; at low oxygen tensions, it acts as a **kinase** on **FixJ**, which regulates expression of two more transcriptional regulators:

NifA, the upstream activator of nif and some fix genes;

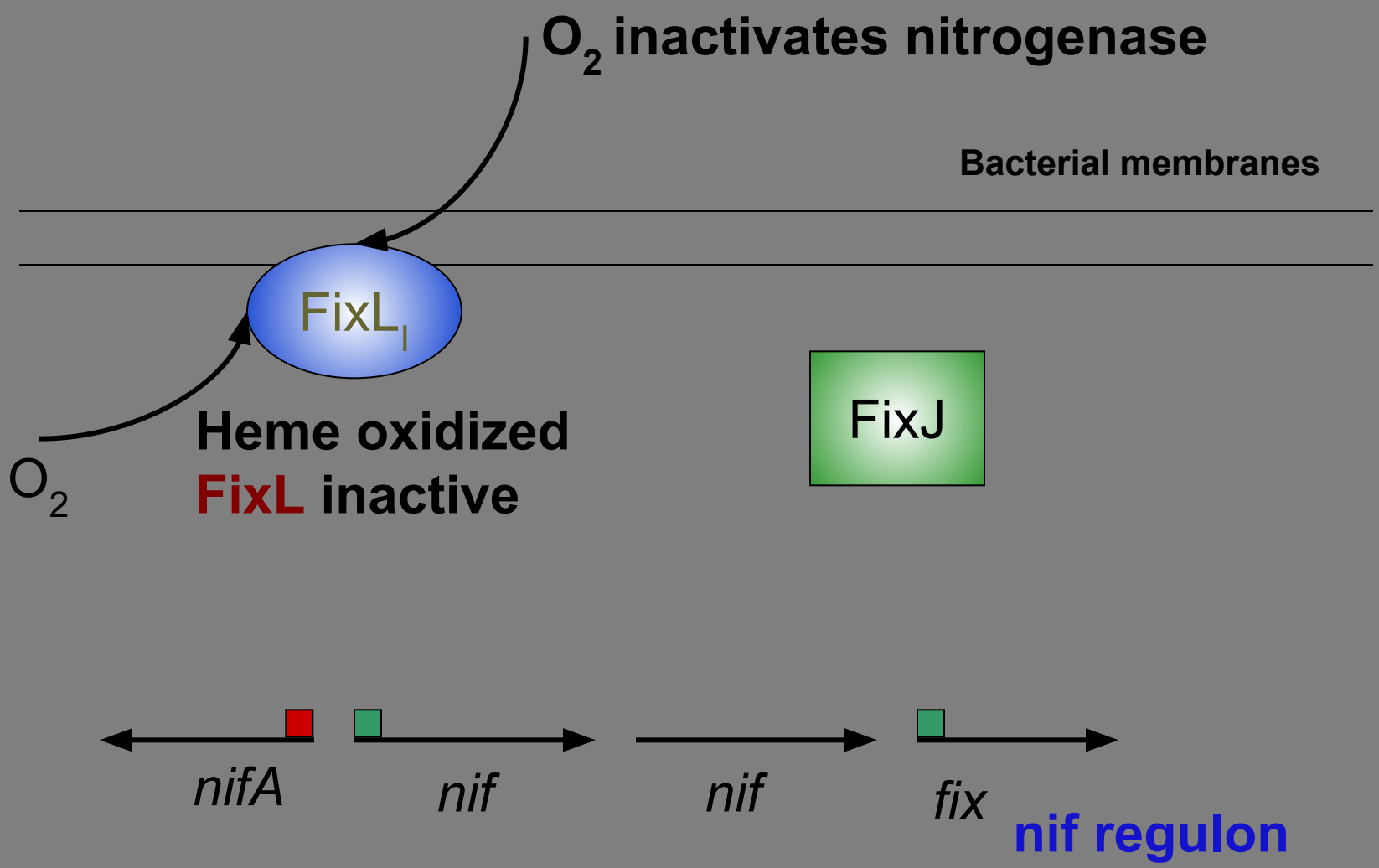
FixK, the regulator of *fixN* (another oxgen sensor?)

This key transducing protein, FixL, is a novel hemoprotein kinase with a complex structure. It has an N-terminal membrane-anchoring domain, followed by the heme binding section, and a C-terminal kinase catalytic domain.

Result?

Low oxygen tension activates nif gene transcription and permits the oxygen-sensitive nitrogenase to function.

Nitrogen fixation genes are repressed by oxygen



Metabolic genes and transporters

Dicarboxylic acid (malate) transport and metabolism

Genes for other functions yet to be identified….

⇒ DNA microarray analysis of gene expression patterns

⇒ Proteomic analysis of bacteroids and peribacteroid membrane preparations

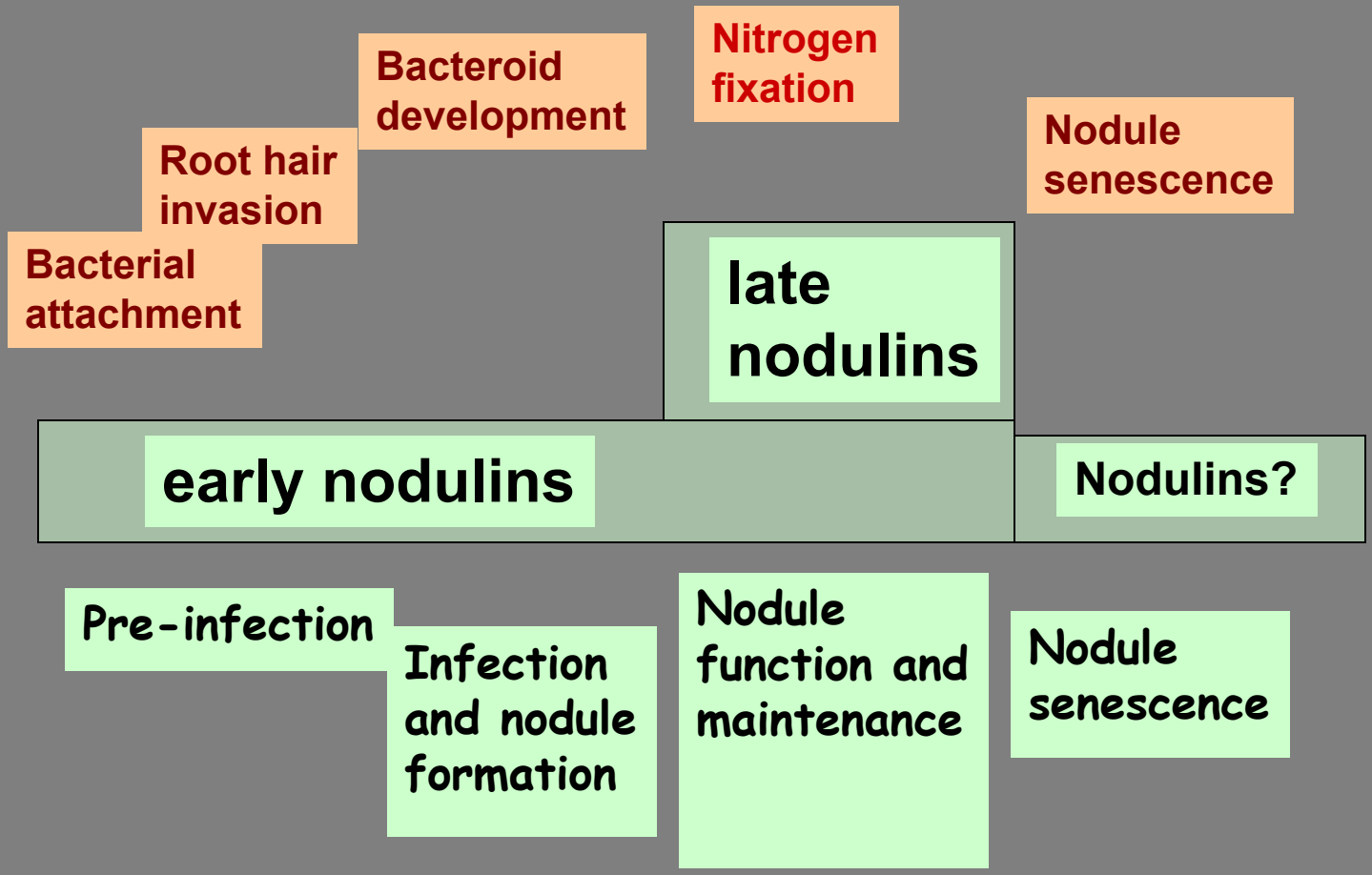
Host plant role in nodulation

1. Production and release of nod gene inducers
   - **flavonoids**

2. Activation of plant genes specifically required for successful nodule formation - **nodulins**

3. Suppression of genes normally involved in repelling microbial invaders - **host defense genes**

Nodulins


Bacterial attachment
Root hair invasion
Bacteroid development
Nitrogen fixation
Nodule senescence
late nodulins
early nodulins
Nodulins?
Pre-infection
Infection and nodule formation
Nodule function and maintenance
Nodule senescence

Early nodulins

At least 20 nodule-specific or nodule-enhanced genes are expressed in plant roots during nodule formation; most of these appear after the initiation of the visible nodule.

Five different nodulins are expressed only in cells containing growing infection threads.
These may encode proteins that are part of the plasmalemma surrounding the infection thread, or enzymes needed to make or modify other molecules

Twelve nodulins are expressed in root hairs and in cortical cells that contain growing infection threads. They are also expressed in host cells a few layers ahead of the growing infection thread.

Late nodulins

The best studied and most abundant late nodulin is the protein component of **leghemoglobin**.
The **heme** component of leghemoglobin appears to be synthesized by the bacteroids.

Other **late nodulins** are enzymes or subunits of enzymes that function in nitrogen metabolism (glutamine synthetase; uricase) or carbon metabolism (sucrose synthase). Others are associated with the peribacteroid membrane, and probably are involved in transport functions.

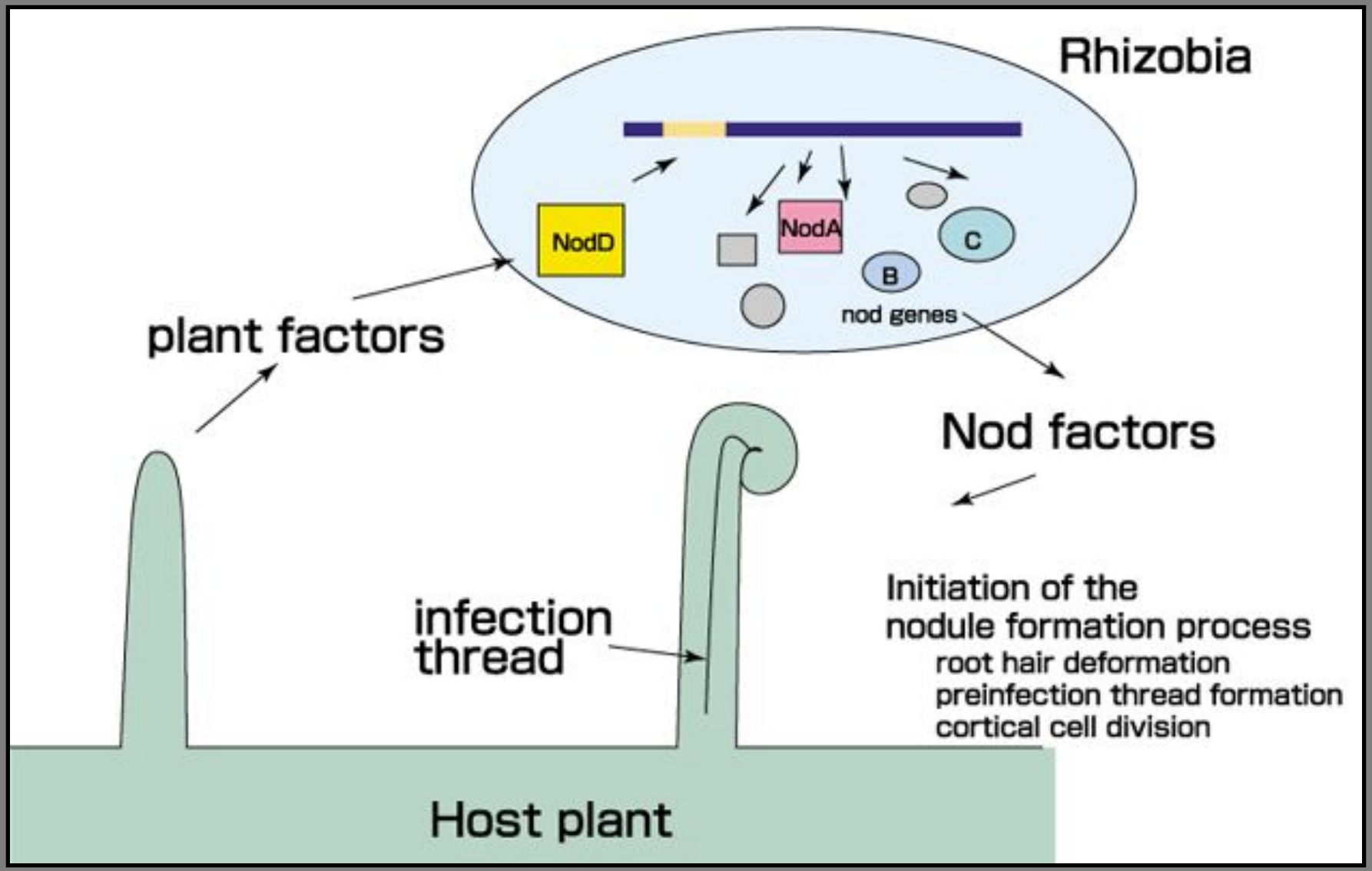
These late nodulin gene products are usually not unique to nodule function, but are found in other parts of the plant as well. This is consistent with the hypothesis that nodule formation evolved as a specialized form of root differentiation.

There must be **many other host gene functions** that are needed for successful nodule formation.

Example: what is the **receptor** for the nod factor?

These are being sought through genomic and proteomic analyses, and through generation of plant mutants that fail to nodulate properly

The full genome sequencing of *Medicago truncatula* and *Lotus japonicus* , both currently underway, will greatly speed up this discovery process.


Rhizobia
NodD
NodA
C
B
nod genes
plant factors
Nod factors
infection
thread
Initiation of the
nodule formation process
root hair deformation
preinfection thread formation
cortical cell division
Host plant

A plant receptor-like kinase required for both bacterial and fungal symbiosis

S. Stracke *et al* Nature 417:959 (2002)

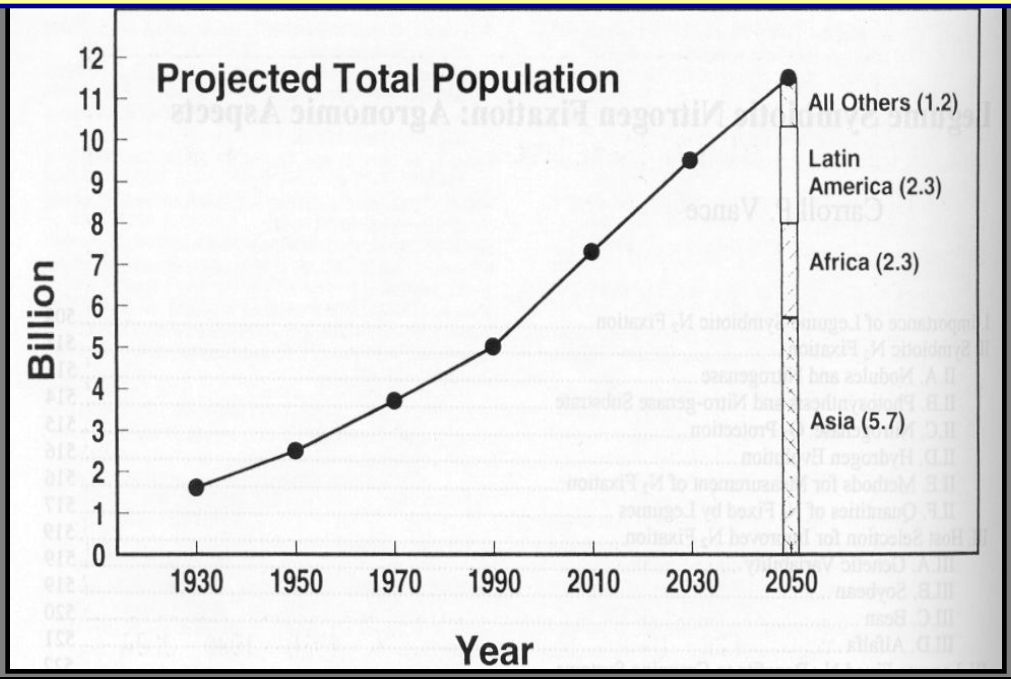
Screened mutagenized populations of the legume *Lotus japonicus* for mutants that showed an inability to be colonized by VAM

Mutants found to also be affected in their ability to be colonized by nitrogen-fixing bacteria ("symbiotic mutants")

Stem-nodulating bacteria

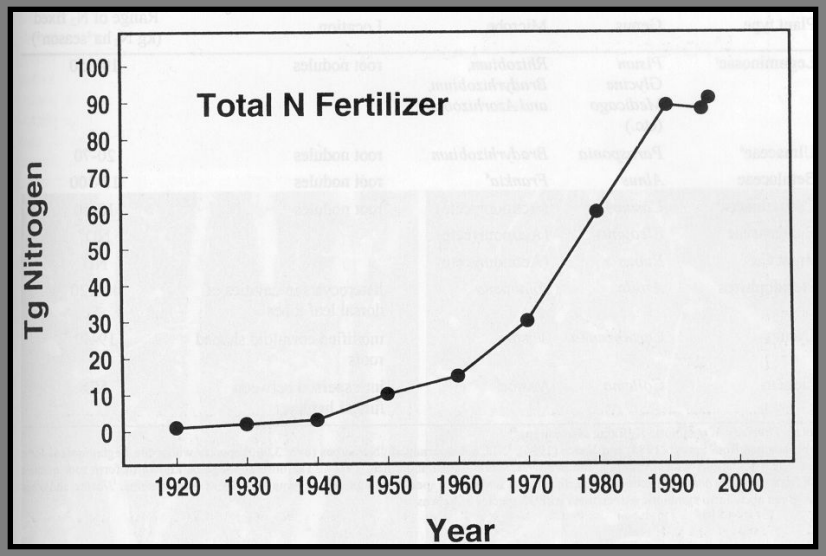
- observed primarily with tropical legumes

A growing population must eat!



- **Combined nitrogen is the most common limiting nutrient in agriculture**
- **Estimated that 90% of population will live in tropical and subtropical areas where (protein-rich) plant sources contribute 80% of total caloric intake.**
- **In 1910 humans consumed 10% of total carbon fixed by photosynthesis, by 2030 it is predicted that 80% will be used by humans.**

Why chemical fertilizers aren't the answer



The Haber-Bosch process

$N_2 + H_2 \longrightarrow 2NH_3$

300 to 1000 bar pressure

400 to 600 C

Catalyst

Consumes 1.4% of total fossil fuels annually

Electrical discharge

$N_2 + O_2 + \text{lightning} \longrightarrow NO_x$

$NO_x + H_2O \longrightarrow HNO_2 \text{ or } HNO_3$

- **Production of nitrogenous fertilizers has "plateaued" in recent years because of high costs and pollution**
- **Estimated 90% of applied fertilizers never reach roots and contaminate groundwater**

Current approaches to improving biological nitrogen fixation

1. Enhancing survival of nodule forming bacterium by improving competitiveness of inoculant strains

2. Extend host range of crops, which can benefit from biological nitrogen fixation

3. Engineer microbes with high nitrogen fixing capacity

What experiments would you propose if you were to follow each of these approaches?