SECTION I

General Microbiology of Rhizobia

CHARACTERISTICS OF THE RHIZOBIA

Rhizobia (the fast-growing *Rhizobium* spp. and the slow-growing *Bradyrhizobium* spp.) or root nodule bacteria are medium-sized, rod-shaped cells, 0.5–0.9 μm in width and 1.2–3.0 μm in length. They do not form endospores, are Gram-negative, and are mobile by a single polar flagellum or two to six peritrichous flagella. Uneven Gram staining is frequently encountered with rhizobia, depending on the age of the culture. Cells from a young culture and nodule bacteroids usually show even Gram staining while older and longer cells give a banded appearance with unstained areas. These unstained areas have been identified to be large granules of polymeric beta-hydroxybutyric acid (PHBA). The PHBA is refractile under phase-contrast microscopy. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino compounds. With the exception of a few strains, they have not been found to fix N in the free-living form except under special conditions. Some strains of rhizobia require vitamins for growth. Bradyrhizobia isolated from soybean (*Glycine max*) and cowpea (*Vigna unguiculata*) nodules were found to remain viable and able to rapidly nodulate their respective host legumes after being stored in purified water at ambient temperatures for periods of at least 1 year. However, *Rhizobium* spp. are likely to lose viability rapidly in water. Optimal growth of most strains occurs at a temperature range of 25–30°C and a pH of 6.0–7.0. Despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions at O_2 tensions of less than 0.01 atm. Generally, most rhizobia produce white colonies, but those that nodulate *Lotononis bainesii* produce a characteristic red nonheme carotenoid pigment when cultured in yeast-mannitol (YM) medium. Most rhizobia only weakly absorb congo red (diphenyldiazo-bis-α-naphthylaminesulfonate) dye, which is included in culture media for isolating rhizobia. However, if the culture medium is not buffered, acid-producing rhizobia cause the dye to turn purple. Other interesting and useful characteristics of rhizobia are their growth reactions in the standard YM medium containing bromthymol blue as the pH indicator. Fast-growing rhizobia produce an acid reaction in the YM medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction.

FREE-LIVING RHIZOBIA IN THE SOIL

Rhizobia are facultative microsymbionts that live as normal components of the soil microbial population when not living symbiotically in the root nodules of the host legume. Outside the root nodule, rhizobia are mostly found on the root surface (rhizoplane), soil around and close to the root surface (rhizosphere), and, to a lesser extent, nonrhizosphere soil. The increase in numbers of rhizobia in the rhizosphere is a response to the excretion of nutrients by plant roots, especially the host legume. Besides the host legume, nonlegumes, moisture and temperature, soil acidity and alkalinity, and salt content of the soil affect rhizobial populations in the soil. Numbers of rhizobia in the soil can range from undetectable to 1,000,000 rhizobia g^{-1} soil. An indirect counting procedure based on plant infection and application of a most-probable-number (MPN) estimate determines the population of rhizobia in the soil . The soil is the major reservoir of free-living rhizobia and under dense or pure stands of legumes, multiplication in the rhizosphere and release of rhizobia from the senescing nodules recolonizes the soil. The various species of rhizobia are not found universally in all soils, but where they are absent, these bacteria may be introduced by seed or soil inoculation. Diverse gene pools of indigenous rhizobia are most likely to be found in the centers of diversity of the host legume. Soil rhizobia are susceptible to attack and lysis by specific bacterial viruses (bacteriophages). Rhizobial phages are as widespread as their host rhizobia and occur widely in soils. The Gram-negative *Bdellovibrio* can parasitize free-living rhizobia. These small, short-curved vibrios are obligate parasites that attach themselves to the larger rhizobial cell and live at the expense of the host.

Rhizobia are somewhat unique among soil microorganisms in their ability to form N_2-fixing symbioses with legumes and, exceptionally, a nonlegume (*Parasponia*). To enjoy the benefits of this partnership, any introduced rhizobia must not only exhibit saprophytic competence among other soil microorganisms, but they must out-compete other rhizobia for infection sites on legume roots. Therefore, potential for physiological versatility is an important trait contributing to their adaptation to the competitive and complex soil environment.

RHIZOBIA AS SYMBIONTS

The free-living rhizobia in the soil can enter the root hairs of the susceptible host legume by a complex series of interactions known collectively as the infection process. This begins with the adhesion of the specific rhizobia to the surface of the root hair. Adhesion is followed by deformation, and curling of the root hair, which results in the characteristic shepherd's crook appearance. The hypha-like infection thread develops gradually in the root hair as a tubular structure that is actually an invagination of the root hair wall. The infection thread contains large numbers of rhizobial cells, and the thread branches through the root cortex passing close to the host cell nuclei. The rhizobia are released from the tip of the infection thread into the cytoplasm of the host cells, where multiplication takes place. Before the release of the rhizobia, rapid host cell division takes place. The dividing host cells are tetraploid. The final structure is a central core containing the rhizobia and a cortical area that becomes occupied by the vascular system, which connects to the young root. The host cell membrane, which had enclosed the infection thread, buds off vesicles containing the rhizobia. The rhizobia divide and differentiate into the form known as bacteroids. The host cell membrane, now referred to as the peribacteroid membrane and the bacteroids, together form the peribacteroid unit. The peribacteroid membranes effectively separate the bacteroids from the plant cytoplasm and provide the plant with a means of regulating nutrient exchange with the bacteroids.

An infected cell from a nodule of a mature soybean plant may contain up to 10,000 peribacteroid units. The forms of bacteroids encountered in the nodules of legumes vary considerably. Branched rods (X- and Y-shaped) and large pear-shaped rounded forms are found in the nodules of *Medicago* spp., *Pisum* spp., and several other species. Perfectly spherical bacteroids are common in nodules of peanuts (*Arachis hypogaea*). The plant largely determines the size and shape of bacteroid, and the numbers in each peribacteroid unit. The synthesis of a protein called *leghemoglobin* in the nodule tissue characterizes effective symbiosis. The presence of leghemoglobin gives a pink/red color to the nodule interior. However, leghemoglobin is absent or present in small quantities in ineffective nodules that appear white when sliced open. The synthesis of leghemoglobin requires genetic information from the legume and the rhizobia.

The enzyme nitrogenase is a complex of two enzymes, an Fe-containing protein and an Fe-Mo protein. It is responsible for the conversion (reduction) of atmospheric N into NH_4^+, and is synthesized in the cytosol of the bacteroids. The legume utilizes NH_4^+ to convert certain precursor metabolites (e.g., α-ketoglutarate, phosphoenopyruvate) into amino acids, which, in turn, are synthesized into proteins. The complex biochemical reactions whereby the inert atmospheric nitrogen is enzymatically reduced into a utilizable form for the plant by the nitrogenase enzyme complex of the bacteroids is called biological nitrogen fixation (BNF).

CLASSIFICATION OF THE RHIZOBIA

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria that are nevertheless classified together by virtue of their ability to nodulate members of the *Leguminosae*. The *Leguminosae* is divided into three subfamilies: *Caesalpinoideae*, *Mimosoideae*, and *Papilionoideae*.

The *Caesalpinoideae* consist mostly of woody plants that show nodulation in a very small number of species. The genus *Chamaecrista* of the *Caesalpinoideae* is well nodulated while nodulation has not been observed in the genera *Delonix*, *Tamarindus*, *Peltophorum*, and many others. The genus *Cassia* is also interesting. *Cassia* spp. grow well in poor soils, but nodulation has not been observed in most of them. *C. leschenaultiana*, found in Hawaiian soils, nodulates with some bradyrhizobia.

The *Mimosoideae* consist mostly of woody species and nodulation occurs at a higher frequency than in the *Caesalpinoideae*. Important genera in this subfamily include *Leucaena* spp., *Acacia* spp., and *Prosopis* spp. Species in certain genera are nodulated by *Rhizobium* and *Bradyrhizobium*. For example, *A. senegal*, *A. farnesiana*, and *A. pennatula* are nodulated by *Rhizobium* while *A. mearnsii*, *A. auriculiformis*, *A. mangium*, and *A. albida* are nodulated by *Bradyrhizobium*. Similarly, *Pithocellobium dulce* is nodulated by *Rhizobium* while *P. jiringa* is nodulated by *Bradyrhizobium*.

The subfamily *Papilionoideae* is well studied for nodulation. Most of the genera in this subfamily are nodulated. The present-day classification of rhizobia is based on earlier studies of the symbiosis with members of the *Papilionoideae*.

The ability of certain rhizobia to infect and nodulate particular group(s) of legume species is important in the classification of rhizobia. Rhizobia are generally classified according to a host-based system. In this host-based system, legume(s) have been assembled into cross-inoculation groups, which are useful in organizing the diverse legumes and their rhizobial partners. Essentially, a cross-inoculation group consists of a collection of legume species that will develop effective nodules when inoculated with the rhizobia obtained from the nodules from any member of that legume group. Classification by this system is by no means perfect, due to cross-inoculation(s) with rhizobia from outside the assigned group and failure to cross-inoculate within a group. The system is not a taxonomic one, but has some practical application. Certain legume-rhizobial associations are highly specific while others are promiscuous. For example, the *Cicer arietinum–Rhizobium* sp. symbiosis is highly specific. *C. arietinum* will nodulate effectively with inoculation with rhizobia isolated only from the nodules of *C. arietinum*. Another instance of specificity is between the forage species *Lotononis bainesii* and the red-pigment-producing *Bradyrhizobium* sp. Here, inoculation of *L. bainesii* sp. with the red strain is necessary for effective nodulation of *L. bainesii*.

At the other extreme are legumes where inoculation with a specific rhizobial strain may not be needed for effective nodulation. Examples of unspecialized or promiscuous groups of legumes are widespread among tropical legumes. Notable examples are *Psophocarpus tetragonolobus*, *Vigna* spp., *Crotalaria* spp., *Macroptilium* spp., *Lablab purpureus*, and *Cajanus cajan*.

Rhizobia belong in the family *Rhizobiaceae*, which consist of the following genera: Genus I—*Rhizobium*; Genus II—*Bradyrhizobium*; Genus III—*Agrobacterium*; and Genus IV—*Phyllobacterium*. Only Genera I and II fix N symbiotically in the root nodules of legumes. The species of rhizobia in Genera I and II, and the cross-inoculation groups of legumes nodulated by these rhizobia are summarized in Table I.1.

In Genus I are the fast-growing acid producers that develop pronounced turbidity in liquid media within 2–3 days and have a mean doubling time of 2–4 h. The cells are motile by two to six peritrichous flagella. They can grow on a wide range of carbohydrates, but usually grow best on glucose, mannitol, or sucrose. Rhizobia of this group are generally infective on temperate legumes.

Table I.1 Species of Rhizobia in Genera I and II, and the Cross-Inoculation Groups of Legumes Nodulated by These Rhizobia

Rhizobia	Cross-Inoculation Group	Legumes in Cross-Inoculation Group
Genus I: *Rhizobium*		
Rhizobium leguminosarum bv. viceae	Pea	Peas (*Pisum* spp.), vetches; (*Vicia* and *Lathyrus* spp.); lentils (*Lens esculenta*)
R. leguminosarum bv. trifolii	Clover	Clovers (*Trifolium* spp.)
R. leguminosarum bv. phaseoli	Bean	Common beans (*Phaseolus vulgaris*); scarlet runner bean (*Phaseolus coccineus*)
R. meliloti	Alfalfa	Alfalfa/medics (*Medicago* spp.); sweet clovers (*Melilotus* spp.); fenugreek (*Trigonella foenumgraecum*)
R. loti	Lotus	Trefoils (*Lotus corniculatus* and *L. tenuis*); lupine (*Lupinus densiflorus*); serradella (*Ornithopus sativus*); kidney vetch (*Anthyllis vulneraria*)
R. galegae		Goat's rue (*Galega orientalis*)
R. fredii	Soybean	Soybean (*Glycine max*)
Rhizobium spp.		Leucaena (*Leucaena* spp); *Gliricidia sepium, Sesbania grandiflora, Calliandra callothyrsus, Pithocellobium dulce, Prosopis pallida, P. juliflora, Acacia senegal, A. farnesiana, Robinia pseudoacacia*
Rhizobium sp.	Chickpea	Chickpea (*Cicer arietinum*)
Genus II: *Bradyrhizobium*		
Bradyrhizobium japonicum	Soybean	Soybean (*Glycine max*)
Bradyrhizobium spp.	Cowpea	Pigeon pea (*Cajanus cajan*); peanut/ groundnut (*Arachis hypogaea*); *Acacia mearnsii, A. mangium, A. auriculiformis*; limabean (*Phaseolus lunatus*); winged bean (*Phosphocarpus tetragonoloba*); siratro (*Macroptilium atropupureum*); guar bean (*Cyamopsis tetragonolobus*); cowpea, mungbean, black/green gram, rice bean (*Vigna* spp.), *Desmodium* spp., *Stylosanthes* spp.; hyacinth bean (*Lablab purpureus*)

In Genus II are the slow-growing, alkali-producing rhizobia, collectively known as bradyrhizobia. They require 3–5 days to produce moderate turbidity in liquid media and have a mean doubling time of 6–8 h. Most strains in this group grow best with pentoses as their C source. The cells are motile by a single polar or subpolar flagellum. A large genera of tropical legume species are nodulated by bradyrhizobia.

Classification of rhizobia is becoming increasingly complex and is revised periodically because of new findings that propose new genera and new species. For example, soybeans are now known to be nodulated by a distinct group of fast-growing, acid-producing rhizobia. These rhizobia were classified in Genus I as *Rhizobium fredii*, but have now been renamed *Sinorhizobium fredii*. More recently, *R. huakuii* has been proposed for a new species of *Rhizobium* that nodulates *Astragalus sinicus*, a legume that is used as green manure in South China. In the bean group, *R. tropicii* has been proposed as a novel species nodulating *P. vulgaris* and *Leucaena* sp. A highly specific symbiosis is established between a specialized strain of fast-growing rhizobia and the tree legume, *Sesbania rostrata*. The nodules are formed on the stems of *S. rostrata* and the nodulating rhizobia have been named *Azorhizobium caulinodans*. A new genus named *Photorhizobium* has been proposed to a group of photosynthetically active rhizobia isolated from the stem nodules of *Aeschenomene*. Also, in the revised classification, *R. trifolii*, *R. phaseoli*, and *R. leguminosarum* are recognized as one species and designated *R. leguminosarum* consisting of three biovars, namely trifolii, phaseoli, and viceae. *R. meliloti* remains as before and *R. loti* has been assigned to the fast-growing *Lotus* rhizobia. Genetically related to *R. loti* are rhizobia from *Lotus corniculatus*, *Lotus tenuis*, *Cicer arietinum*, *Leucaena leucocephala*, and *Sophora microphylla*. Rhizobia nodulating *Vigna*, *Arachis*, *Desmodium*, *Macroptilium*, *Stylosanthes*, and many other tropical legumes are still unclassified at the species level, but are grouped as *Bradyrhizobium* spp. The non-legume *Parasponia* (previously called *Trema*) is also nodulated by a *Bradyrhizobium* sp. Besides *Leucaena*, there are other legumes (e.g., *Sesbania*, *Neptunia*, *Calliandra*, and *Acacia*) that are nodulated by fast-growing, acid-producing rhizobia. The taxonomic status of these organisms needs to be resolved in the future.