PLANT-ASSOCIATED BACTERIA

Plant-Associated Bacteria

Edited by

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PREFACE

The idea of developing a comprehensive volume on PLANT-ASSOCIATED BACTERIA was born in my mind about 5 years back. I decided therefore that the proposed volume on Plant-Associated Bacteria will be a complete volume on plant bacteriology. There are several books on symbiotic and beneficial bacteria. There are also books and volumes on plant pathogenic bacteria. In recent years, the plant-growth promoting rhizobacteria (PGPR) has emerged as an important group which has significant applications to crop production and biological disease control. There is a vital need for a comprehensive volume on all plant-associated bacteria that also includes epiphytic and endophytic bacteria. In my mind, the proposal appeared worthwhile and timely.

In the present era of genomics, there has been an enhanced interest in the genomics of the plant-associated bacteria (Example: the Plant-Associated Microbe Genome Initiative, American Phytopathological Society–www.apsnetorg/ media/ ps/top.asp). Traditional methods of identification of bacteria have been replaced by molecular methods for diagnostics and their phylogeny. This volume tries to carefully document both the traditional and recent methods. To achieve this, the contributors have been chosen from among the best experts to provide updated information for a reader who has specific expectations in a volume of this nature. Today, readers want specific and detailed information in a narrow area and there are those who want information on broad themes with adequate information on all different groups of bacteria with links and citations. There are several volumes (perhaps there are several others in the making) which could fulfill the first category. This volume belongs to the second category, a resource manual and justifiably, a reference volume.

It is hard to find volumes on broad themes. The volume of Plant-Associated Bacteria has been designed to cover the whole theme of plant bacteriology. Recent advances on all the different groups of bacteria that are associated with the phylloplane and rhizosphere have been dealt with and described whether they are beneficial (symbiotic/diazotrophic), epiphytes, endophytes or pathogens of the plants. It is my hope that a reader who is looking for information either on beneficial or pathogenic bacteria of plants, he/she finds the information in this volume with adequate details. If the reader is a researcher who needs detailed coverage of individual groups of bacteria, their strains, molecular biology and improvement, the volume devotes substantial portions of information on each aspect. This volume can be a reference material which chronicles both the traditional information on aspects such as methods of identification and the new/recent molecular methods. Therefore, it should fill the need for a good resource volume both for research students and scientists.

SAM S. GNANAMANICKAM
Editor

ACKNOWLEDGEMENT

I am deeply grateful to all the contributors. Each one brings a great deal of expertise and yet it was a very cordial experience in relating to work that went into developing this volume by assembling eighteen contributions made by these able men and women.

I am very thankful to my research associates who took a heavy share of the work. In particular, Dr. Boney Kuriakose, Dr. V. Arun and Dr. J. Ebenezar Immanuel are acknowledged for the help they rendered.

A great deal of encouragement was afforded to me by Dr. Gwyn Beattie, Dr. Tim Denny and Dr. Cindy Morris. I appreciate these sources of strength and encouragement.

Dr. Hari B. Krishnan USDA-ARS at U. of Missouri-Columbia readily lent his picture that is featured on the book's cover to represent a nodulating *Sinorhizobium* on *Erythrina* sp. I am thankful for this courtesy.

GWYN A. BEATTIE

PLANT-ASSOCIATED BACTERIA: SURVEY, MOLECULAR PHYLOGENY, GENOMICS AND RECENT ADVANCES

Abstract. Bacteria that associate with plants are diverse in the habitats they occupy, their phylogeny, and their effects on plant and environmental health. The spermosphere, rhizosphere, phyllosphere, vascular tissue and endophytic regions offer distinctive habitats for bacteria. The phylogenetic diversity of bacteria in these habitats extends across the Bacterial and Archaeal Domains; however, bacteria that are known phytosymbionts or phytopathogens are classified into only four Bacterial phyla, the Cyanobacteria, Proteobacteria, Firmicutes, and Actinobacteria. Most plant-associated prokaryotes are commensals that have no detectable effect on plant growth or physiology; these are found primarily on plant surfaces. Mutualistic bacteria include the legume symbionts, *Frankia*, and cyanobacterial symbionts, which form nitrogen-fixing symbioses, as well as associative nitrogen-fixing bacteria and plant growth-promoting rhizobacteria, which can enhance plant growth directly by increasing nutrient availability or producing plant growth-enhancing products, and indirectly by biologically controlling plant diseases. Phytopathogenic bacteria are diverse in the symptoms they induce as well as in their invasion strategies, mechanisms of pathogenesis, culturability, and even genome structure and fluidity. Other organisms that can detrimentally influence plant health are the ice nucleating bacteria, bacteria that overproduce plant growth regulators, and the deleterious rhizobacteria, which can help control weeds. Effects of plant-associated bacteria on environmental health include contributions to the remediation of soilborne pollutants, the decomposition of organic matter, and soil aggregation. Recent advances in genomic sequencing, functional genomics, and microbial ecology are dramatically changing our research approaches to these organisms and the questions we can address about their biology and their interactions with plants.

1. INTRODUCTION

Bacteria that associate with plants are diverse in their ability to affect plant health, their genotypic and phenotypic characteristics, and their phylogeny. These bacteria are typically members of complex microbial communities, with only a few establishing pure clonal populations within a plant. Although the majority of research on plant-associated bacteria has focused on phytopathogens and diazotrophic (nitrogen-fixing) phytosymbionts, interest in the diversity of organisms associated with plants has increased as the tools to assess diversity have advanced. It is clear that many plant-associated microbes, even those that comprise only a small proportion of a community, can have functions that are of agricultural or environmental importance. Technical advances in microbial ecology and genomics have been paralleled by advances in our understanding of the structure and dynamics of these microbial communities and in the molecular basis of plant-microbe and microbe-microbe interactions. The aim of this book is to provide comprehensive coverage of all types of bacteria that are found in association with plants.

S.S. Gnanamanickam (ed.), Plant-Associated Bacteria, 1-56.

2. OVERVIEW OF PLANT-ASSOCIATED BACTERIA AND THEIR HABITATS

Prokaryotes, and primarily members of the Bacterial Domain, are the numerically dominant component of most microbial communities on plants. These prokaryotes, collectively referred to as bacteria, can attain densities as high as 10^9 cells per gram of plant tissue of roots based on culturing, and 10^{10} cells per g based on cultivation-independent methods. The eukaryotic microflora can include filamentous fungi, yeasts, algae, protozoa, and nematodes, but usually at densities many orders of magnitude lower than the prokaryotes. For example, the rhizosphere may contain, in culturable numbers per gram of root, 10^6-10^9 non-actinomycetous bacteria, 10^7 actinomycetes, 10^5-10^6 fungi, 10^3 algae and 10^2-10^3 protozoa. Bacteriophage and viruses can also be members of these communities and, although the populations in the rhizosphere have not been extensively examined, they have been found at densities as high as 10^8 to 10^9 per gram of soil (Ashelford et al., 2003; Williamson et al., 2005). Although many interesting interactions occur within these communities, and some are being exploited for the control of plant pathogens, this chapter is focused exclusively on the associations between bacteria and their plant hosts.

2.1. Plants as habitats for bacteria

Plants offer a wide range of habitats that support microbial growth. These include sites that are moist and rich in nutrients, and thus ideal for fostering bacterial growth, as well as sites that are nutrient-poor or exposed to stressful environmental conditions. The surfaces of seeds, roots, leaves, and fruits often harbor large, diverse bacterial communities, whereas blossoms, stems, vascular tissue, and the intercellular spaces within plant tissues often are free of microorganisms or support only limited bacterial communities.

2.1.1. The spermosphere

The *spermosphere* is the zone that is influenced by a seed; it often extends 1 to 10 mm from the seed surface. Nutrients that support microorganisms are released when the seed imbibes, with nutrient release being greatest from the embryo end, i.e., the end closest to the emerging radicle, and from seeds that are cracked or damaged. Bacteria that establish populations on seeds can colonize roots as they emerge.

2.1.2. The rhizosphere

The *rhizosphere* is the zone that is influenced by the root, although experimentally it is often defined as the soil adhering to the root. Root growth changes the physical and chemical properties of the soil, including the mineral and organic content, the water potential, the pH, and the salinity. Whereas most soils are low in nutrients, the rhizosphere is nutrient rich. This region contains root exudates (low molecular weight (MW) compounds released during normal root cell metabolism), root secretions (low and high MW compounds that are synthesized for secretion), and compounds released by plant cell lysis. A major component of root secretions is mucilage, which contains hydrated polysaccharides, organic acids, vitamins, and amino acids and thus is an excellent substrate for microbial growth. Mucilage binds water and thus helps to form a well-hydrated environment for the roots and rhizosphere microorganisms. During root growth, the root cap continuously sloughs off cells, called peripheral or border cells, that secrete large amounts of mucilage to lubricate the roots, and after living for about 3 weeks, die and lyse. These cells are thus a major source of the mucilage and lysates available to microbial communities in the rhizosphere. The root tip itself is usually devoid of microorganisms due to the rapid rate at which border cells are shed and the rapid growth of roots, which together prevent the continuous presence of bacteria at the root tip. In contrast, the region between the root cap and the mature root, where the border cells accumulate, supports large communities. The epidermal cells in this region release additional exudates and secretions during cell division, elongation, and differentiation into root hairs. Emerging lateral roots promote the release of even more nutrients via plant cell lysis. Because of the outward diffusion of nutrients and the inward movement of salts and minerals during transpiration, chemical gradients form around the root and create a range of distinct microbial habitats. Furthermore, mature roots produce less mucilage and fewer cell lysates, due to the absence of border cells and emerging lateral roots, and leak less water due to the deposition of a water-impermeable suberin layer around epidermal cells. Consequently, the developing roots generally support fast-growing microorganisms like bacteria, whereras mature roots support slower-growing microorganisms like fungi and actinomycetous bacteria.

2.1.3. The phyllosphere

The external regions of the above-ground parts of plants, including leaves, stems, blossoms, and fruits, are collectively referred to as the *phyllosphere.* Leaves are the dominant tissue in the phyllosphere based on the surface area available for colonization. The waxy plant cuticle ensures that water loss occurs primarily through the stomata, an adaptation that probably evolved to allow plants to live on land and that has major implications for the leaf surface microflora. Unlike the rhizosphere, the phyllospehre is subject to large and rapid fluctuations in temperature, solar radiation, and water availability, the latter of which increases with rain, dew, or fog and usually decreases with wind. These changes in environmental conditions are somewhat buffered by the *boundary layer,* an air layer that surrounds the leaf. In contrast to the rhizosphere, which often supports at least 10^9 bacteria per gram of root, leaf surfaces typically support fewer than 10^7 bacteria per gram of leaf, although these community sizes vary greatly with plant species, physiology, age, and environmental conditions. Plant-derived nutrients on leaves probably originate from mesophyll and epidermal cell exudates leaking onto the surface as well as lysates from wounds and broken trichomes. Several lines of evidence indicate that the distribution of these nutrients is highly heterogeneous (Leveau & Lindow, 2001), as would be predicted for those originating from leaf damage or exogenous sources such as the honeydew of visiting insects. Blossoms and fruits offer unique habitats for microbial growth. Blossoms are short-lived, contain sugar-rich nectar (e.g., 10-50% sucrose), and are attractive to insects, which are excellent vectors for bacteria. Fruits generally have a thick cuticle on their epidermis, which probably minimizes the leakage of water and nutrients to the fruit surface.

2.1.4. Endophytic sites

Endophytic sites include any region internal to the plant epidermis, although the vascular system is usually considered separately. Endophytic microorganisms are usually found within the intercellular, or apoplastic, spaces. Intercellular air spaces comprise a significant fraction of the tissue inside roots and leaves. For example, the spaces between the cortical root cells can comprise as much as 30% of the root volume, and those between the mesophyll leaf cells can comprise as much as 70% of the leaf volume. Microorganisms that reach these intercellular regions must contend with plant defense responses, which are triggered when bacteria are in close proximity to the plant cells. Bacteria in endophytic sites may access nutrients and water more easily than those on the surface, particularly if the plant cells lyse or leak nutrients, as occurs during pathogenesis. Bacteria in endophytic sites may be buffered from the environmental fluctuations characteristic of the phyllosphere and from the intense competition for nutrients characteristic of the rhizosphere. Bacterial entry into plants occurs at sites of epidermal damage, of lateral root or radicle emergence, through natural openings such as stomata and lenticels (pores for gas exchange), hydathodes (water pores), nectarthodes (openings in the nectary of blossoms), and into progency plants through infected seeds. Some symbiotic bacteria have evolved sophisticated entry mechanisms that include directing the plant to form a channel, called an infection thread, that promotes bacterial penetration into the plant tissue.

2.1.5. Vascular tissue

The two elements of the vascular system, the xylem and the phloem, offer distinct habitats for microbial colonization. Xylem vessels function in the transport of water and minerals and thus contain a highly dilute solution of minerals and simple organic compounds. The vessels consist of dead cells that do not contain cytoplasm, lignified secondary cell walls, and lateral wall openings, called pits, with membranes that must be crossed to leave the xylem. Some bacterial xylem colonists can live on only the nutrients in the xylem; these fastidious organisms exhibit complex requirements for growth indicative of a high level of adaptation to this habitat. Others live on nutrients that are released following destruction of the xylem vessel walls. In contrast, the phloem functions in the transport of sugars from the leaves to the rest of the plant and contains a concentrated sucrose solution (15-30%). The phloem consists of living cells, including sieve tube elements and their associated companion cells, which load sucrose into the sieve tubes. The plates between adjacent sieve elements have pores with a diameter of 1 to 15 μm, which is large enough to allow bacterial passage. Bacteria that can colonize the phloem are highly adapted to this habitat, as evidenced by the low cultivation rate of these organisms and the unique presence of phytoplasmas, or cell wall-less bacteria, in the phloem. Phloem colonists, all of which are believed to be pathogens, are also unique among plant-associated bacteria in being located intracellularly. Bacteria gain access to the xylem tissue through a variety of routes, including through stomata or hydathodes that lead to the open ends of xylem vessels in leaves, through the sites of lateral root emergence in roots, through xylem-feeding insects and wounds, and via active dissolution of the xylem vessel walls. In contrast, bacteria gain access to the phloem primarily by transmission from phloem-feeding insects or by cultivation practices such as grafting, which involves cutting stems.

2.2. Current bacterial taxonomy

Prokaryotes that live in the multitude of habitats in and on plants are classified into a limited number of taxonomic groups. The current taxonomic outline of the prokaryotes, as presented in *Bergey's Manual of Systematic Bacteriology*, is shown in Fig 1. At present, all named prokaryotes fall within 2 phyla that comprise the Archaeal Domain and 24 phyla that comprise the Bacterial Domain. This outline requires continuous updating because of the current pace of change in bacterial taxonomy. These changes are fueled by improved methods for characterizing cultivated organisms, particularly at the genotypic and molecular level, as well as exponential increases in the discovery of new organisms that have not yet been cultivated. Uncultivated organisms are included in the taxonomic overview compiled by *Bergey's Manual of Systematic Bacteriology* only when they meet at least a minimal level of characterization criteria and are published in, or approved by, the *International Journal of Systematic and Evolutionary Microbiology.*

The Archaea and the Bacteria are the currently accepted names for the two distinct lineages of prokaryotes. These lineages were deduced primarily based on the sequence of the 16S ribosomal RNA (rRNA) subunit found in all prokaryotic cells. The current, standard polyphasic approach to bacterial taxonomy involves constructing a phylogenetic, or evolutionary, tree based on 16S rRNA sequence information, using it as the initial basis for classification, and then validating the tree multidimensionally by examining many phenotypic characteristics (physiological and biochemical traits), genotypic characteristics (G+C content, DNA-DNA similarity, and DNA-rRNA similarity), and other chemotaxonomic characteristics (e.g., cellular fatty acids). In general, hierarchical classifications at the levels of Domain, Phylum, Class, Order, Family, and Genus are mainly based on phylogenetic analyses using 16S rRNA sequences, whereas classification at the Species level is mainly based on DNA-DNA hybridization analyses. Although the resulting bacterial taxa are not "official" in that an official bacterial classification system does not exist, the taxa that are presented by *Bergey's Manual* represent the best consensus available.

2.3. Plant-bacterial associations

Plant-associated prokaryotes can be grouped based on the nature of their interaction with a host plant. Bacteria that are not known to affect the plant, at least directly, can be classified as *commensals*, those that have a beneficial effect on the plant can be called *mutualists*, and those that have an adverse effect on the plant are usually called *pathogens* or *parasites*. These terms are premised on the assumption that, in all cases, the plant benefits the bacteria by providing nutrients. Although many bacteria are clearly commensals, mutualists or pathogens, others fall along a continuum among these groups (Hentschel et al., 2000; Hirsch, 2004). For example, one species may utilize nutrients only passively released by a plant, another may induce a low level of active nutrient release with no detectable impact on the host, and yet another may induce the release of sufficient nutrients to cause a slight, but detectable, decrease in host fitness. A single organism can be a commensal on one host and a pathogen on another, or a commensal for months preceding entry into a pathogenic phase. All plant-associated bacteria are likely to exhibit a commensalistic relationship with a host at some stage during their lifecycle. Thus, in the following description of plant-associated bacteria, the designation of an organism as a mutualist or a pathogen indicates only the *potential* for an organism to have a mutualistic or pathogenic association with one or more plant species.

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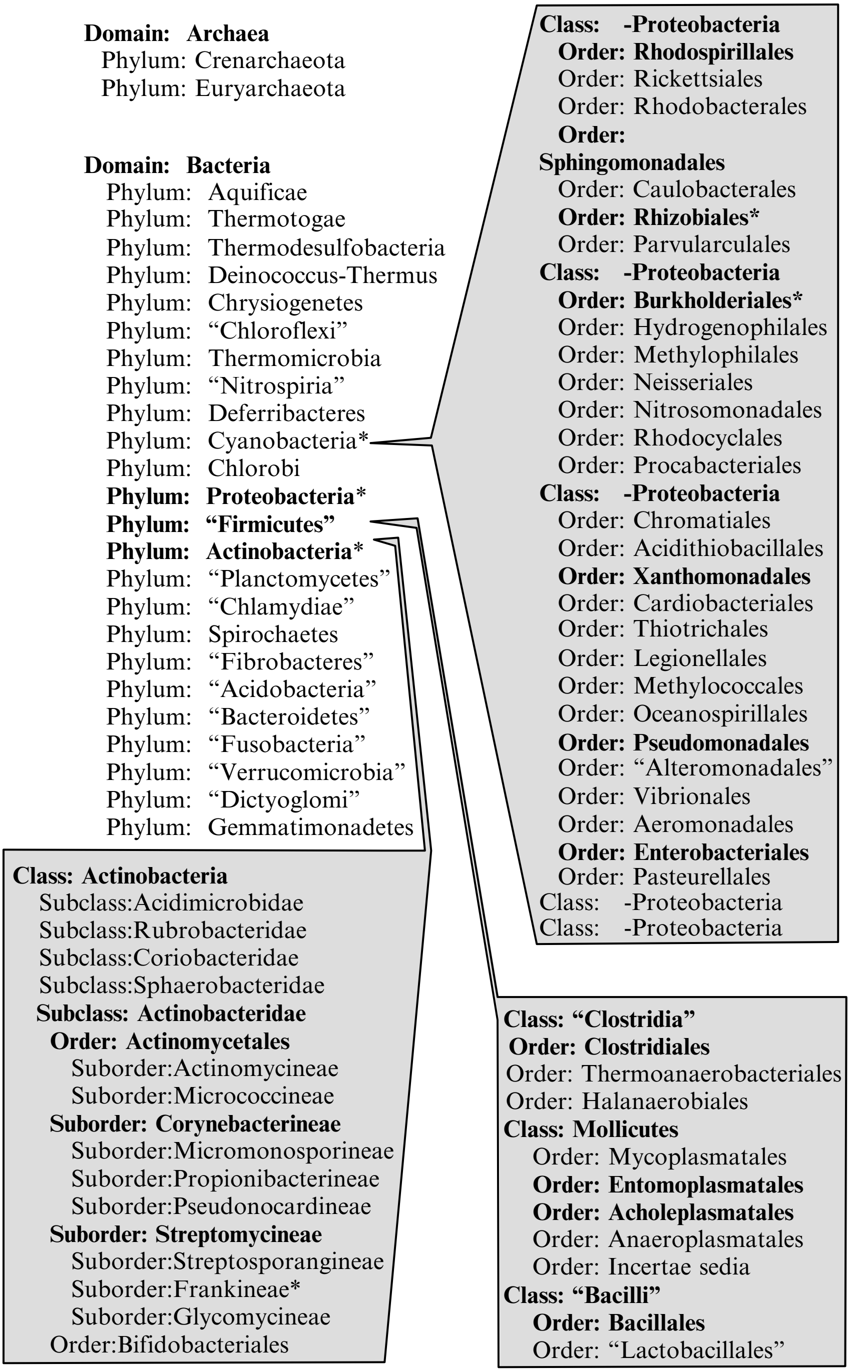
Figure 1. Taxonomic overview of the prokaryotes. Taxonomic classifications within phyla are shown only for those phyla that contain phytopathogens or phytosymbionts. Phytopathogens are found only in those taxa that are in bold; plant symbionts are found only in those taxa indicated with an asterisk. This classification is from Bergey's Manual of Systematic Bacteriology, 2^{nd} Edition, Release 5.0 (March 2004) http://dx.doi.org/10.1007/bergeysoutline200310. The names in quotes have no standing in nomenclature, but are useful as indicators of the taxon.



Domain: Archaea
Phylum: Crenarchaeota
Phylum: Euryarchaeota

Domain: Bacteria
Phylum: Aquificae
Phylum: Thermotogae
Phylum: Thermodesulfobacteria
Phylum: Deinococcus-Thermus
Phylum: Chrysiogenetes
Phylum: "Chloroflexi"
Phylum: Thermomicrobia
Phylum: "Nitrospiria"
Phylum: Deferribacteres
Phylum: Cyanobacteria*
Phylum: Chlorobi
Phylum: Proteobacteria*
Phylum: "Firmicutes"
Phylum: Actinobacteria*
Phylum: "Planctomycetes"
Phylum: "Chlamydiae"
Phylum: Spirochaetes
Phylum: "Fibrobacteres"
Phylum: "Acidobacteria"
Phylum: "Bacteroidetes"
Phylum: "Fusobacteria"
Phylum: "Verrucomicrobia"
Phylum: "Dictyoglomi"
Phylum: Gemmatimonadetes

Class: -Proteobacteria
Order: Rhodospirillales
Order: Rickettsiales
Order: Rhodobacterales
Order: Sphingomonadales
Order: Caulobacterales
Order: Rhizobiales*
Order: Parvularculales
Class: -Proteobacteria
Order: Burkholderiales*
Order: Hydrogenophilales
Order: Methylophilales
Order: Neisseriales
Order: Nitrosomonadales
Order: Rhodocyclales
Order: Procabacteriales
Class: -Proteobacteria
Order: Chromatiales
Order: Acidithiobacillales
Order: Xanthomonadales
Order: Cardiobacteriales
Order: Thiotrichales
Order: Legionellales
Order: Methylococcales
Order: Oceanospirillales
Order: Pseudomonadales
Order: "Alteromonadales"
Order: Vibrionales
Order: Aeromonadales
Order: Enterobacteriales
Order: Pasteurellales
Class: -Proteobacteria
Class: -Proteobacteria

Class: Actinobacteria
Subclass:Acidimicrobidae
Subclass:Rubrobacteridae
Subclass:Coriobacteridae
Subclass:Sphaerobacteridae
Subclass: Actinobacteridae
Order: Actinomycetales
Suborder:Actinomycineae
Suborder:Micrococcineae
Suborder: Corynebacterineae
Suborder:Micromonosporineae
Suborder:Propionibacterineae
Suborder:Pseudonocardineae
Suborder: Streptomycineae
Suborder:Streptosporangineae
Suborder:Frankineae*
Suborder:Glycomycineae
Order:Bifidobacteriales

Class: "Clostridia"
Order: Clostridiales
Order: Thermoanaerobacteriales
Order: Halanaerobiales
Class: Mollicutes
Order: Mycoplasmatales
Order: Entomoplasmatales
Order: Acholeplasmatales
Order: Anaeroplasmatales
Order: Incertae sedia
Class: "Bacilli"
Order: Bacillales
Order: "Lactobacillales"

3. PHYTOBACTERIA WITH A COMMENSALISTIC ASSOCIATION WITH PLANTS

3.1. Rhizosphere and phyllosphere bacteria

The vast majority of plant-associated bacteria live on the nutrients that are leaked onto plant surfaces and do not detectably alter the growth or physiology of the plant; thus, most plant-associated bacteria have a commensalistic association with their host. The distinct habitats on a plant support distinct microbial communities. For example, cultured isolates from leaves are commonly pigmented whereas those from the rhizosphere are not; pigmentation is a trait often associated with tolerance to solar radiation. In general phyllosphere communities are composed of a few taxa that have a relatively large number of individuals and many taxa with a small number of individuals (Hirano & Upper, 2000). For example, among 1,701 cultivated isolates from olive leaves, *Pseudomonas syringae* comprised the majority of the isolates whereas each of the other taxa comprised less than 10% (Table 1), and among isolates from sugar beet leaves that were collected over a complete growing season, no taxon formed a majority (Table 1).

Among the cultivated isolates from leaves, Gram$^-$ bacteria are more common than Gram$^+$ bacteria. The size and composition of phyllosphere communities, however, varies greatly with plant species, leaf physiology, leaf age, and environmental conditions, making it difficult to make generalizations about these communities. As an illustration of their taxonomic diversity, isolates from spring wheat leaves collected over a grown season represented 37 genera and 88 species (Legard et al., 1994). Rhizosphere communities are similarly, or perhaps even more, complex. Microbial communities in the rhizosphere clearly differ in composition from those in non-rhizosphere (bulk) soils, and rhizospere diversity has been found to be influenced by a wide range of factors, including plant species, plant disease, plant nutrition, fertilizer application, soil type, and environmental conditions (Kent & Triplett, 2002).

Cultivation-independent methods for examining microbial communities in natural habitats have suggested that cultivated isolates represent less than 1% of the bacterial taxa present (Torsvik & Øvreås, 2002). The finding that many organisms identified by cultivation-independent methods do not belong to known taxa further suggests that there is a rich, uncultured microbial world to be explored. The richness of this uncultured world is reflected in the fact that including candidate phyla that are monophyletic lineages based on 16S rRNA sequence and that are distinct from the existing phyla would likely double the number of recognized phyla in the domain Bacteria (Rappe & Giovannoni, 2003). It is clear that the breadth of bacterial diversity in the environment is significantly wider than is reflected in our current taxonomic outline (Fig 1). Cultivation-independent methods have indicated that plant-associated communities are more complex than was suggested by the cultivated isolates. For example, of 17 organisms identified as the dominant organisms in a phyllosphere community using cultivation-independent methods, 13 had not been described in previous cultivation-based phyllosphere studies and 5 were novel species (C. H. Yang et al., 2001). A large number of novel species were also found in a microbial community from an aquatic leaf (Weidner et al., 2000). Cultivation-independent methods led to the exciting discovery that members of the Archaeal Domain, and specifically of the phylum *Crenarcheae*, can be found in great abundance on plant roots (Simon et al., 2000). Subsequent studies have shown that roots select for a crenarchael consortium that is distinct from that the bulk soil (Sliwinski & Goodman, 2004).

3.2. Endophytic bacteria

Endophytic bacteria, or microorganisms that live within the tissues of living plants without causing substantive damage to the host. They can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue. Although endophytic fungi have been extensively studied, endophytic bacteria have not. These bacteria, however, are of increasing interest due in part to their potential uses in agriculture

Table 1. Bacterial community composition on leaves.

Olive leaves[1]	%	Sugar beet leaves[2,3]	%
Pseudomonas syringae	51.0	*Pantoea agglomerans*	11.2
Xanthomonas campestris	6.7	*Pseudomonas aureofaciens*	9.6
Pantoea agglomerans	6.0	*Pseudomonas syringae*	6.2
Acetobacter aceti	4.7	*Arthrobacter oxydans*	4.8
Gluconobacter oxydans	4.3	*Raoultella terrigena*	3.2
Pseudomonas fluorescens	3.9	*Kocuria lylae*	2.9
Bacillus megaterium	3.8	*Kurthia zopfi*	2.8
Leuconostoc mesenteroides	3.1	*Aeromonas caviae*	2.7
Lactobacillus plantarum	2.8	*Pseudomonas marginalis*	2.2
Curtobacterium plantarum	2.2	*Serratia liquefaciens*	2.1
Micrococcus luteus	2.2	*Micrococcus kristinae*	2.1
Arthrobacter globiformis	1.4	*Total Bacillus spp.*	2.0
Raoultella planticola	1.2	*Stenotrophomonas maltophilia*	1.9
Enterococcus faecium	1.2	*Pseudomonas putida*	1.8

Clavibacter spp.	0.98	*Microbacterium saperdae*	1.8
Micrococcus spp.	0.82	*Flavobacterium johnsonae*	1.3
Serratia marcescens	0.81	*Arthrobacter globiformis*	1.3
Bacillus subtilis	0.57	*Pseudomonas fluorescens*	1.1
Cellulomonas flavigena	0.40	*Brevundimonas vesicularis*	1.0
Erwinia spp.	0.37	*Pseudomonas pseudoalcaligenes*	0.9
Zymomonas mobilis	0.30	*Curtobacterum flaccumfaciens*	0.9
Bacillus spp.	0.29	*Microbacterium lacticum*	0.8
Alcaligenes faecalis	0.27	*Arthrobacter mysorens*	0.7
Erwinia carotovora	0.08	*Arthrobacter viscosus*	0.7
Pseudomonas aeruginosa	0.04	*Bacillus pumilus*	0.7
		Pseudomonas cichorii	0.6
		Pseuodmonas viridiflava	0.6
		Raoultella planticola	0.6
		Yersina enterocolitica	0.6
		Pseudomonas chlororaphis	0.5
		Pseduomonas mendocina	0.5
		Chryseobacterium indologenes	0.5
		Erwinia rhapontici	0.4
		Hafnia alvei	0.4
		Serratia plymuthica	0.4
		Agrobacterium rubi	0.4

[1] *1,701 isolates from olive leaves were identified based on phenetic traits (Ercolani, 1991)*

[2] *1,236 isolates from sugar beet leaves were identified based on fatty acid methyl ester analysis (Thompson, 1993)*

[3] *Additional species were identified that collectively represented less than 0.4% of the community: Acinetobacter calcoaceticus, Ac. lwoffii, Aeromonas salmonicida, Ae. sobria, Agrobacterium radiobacter, Ag. tumefaciens, Arthrobacter atrocyaneus, Ar. crystallopoietes, Ar. protophormiae, Ar. ureafaciens, Aureobacterium esteraromaticum, Cellulomonas turbata, Chryseobacterium balustinum, Clavibacter michiganensis, Comamonas acidovorans, Com. testosteroni, Corynebacterium bovis, Enterobacter amnigenus, Ent.intermedius, Erwinia chrysanthemi, Hydrogenophaga pseudoflava, Janthinobacterium lividum, Kocuria roseus, Microbacterium barkeri, Mi. liquefaciens, Micrococcus luteus, Morganella morganii, Ochrobacterium antropi, Pseudomonas corrugata, Ps. oryzihabitans, Ps. stutzeri, Rhodococcus rhodochrous, Sphingobacterum*

(Kobayashi & Palumbo, 2000). Endophytic bacteria have been isolated from a wide variety of healthy plant species and tissue types. Kobayashi and Palumbo (2000) compiled a list of 55 bacterial genera and 144 species isolated as endophytes from roots, stems, flowers, tubers, seeds, or fruit of the many plant species examined; this included almost 50 bacterial endophytes that were isolated from only two plant species (McInroy & Kloepper, 1995). Approximately 15% of the endophytes identified could also be classified as phytopathogenic species, but the isolates may represent nonpathogenic strains or may have been present in a host or environmental context that was not appropriate for disease. Some of the most commonly isolated endophytes from the rhizosphere include species of *Bacillus, Enterobacter, Serratia, Stenotrophomonas,* and fluorescent pseudomonads (Kobayashi & Palumbo, 2000), whereas isolates showing promising levels of colonization and persistence in various agronomic crops and prairie plants included species of *Cellulomonas, Clavibacter, Curtobacterium* and *Microbacterium* (Zinniel et al., 2002). All of these isolates fall in the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes phyla of the domain Bacteria (Fig 1).

4. PHYTOBACTERIA WITH A MUTUALISTIC ASSOCIATION WITH PLANTS

Bacteria that can benefit plants have been of great biological and agricultural interest since 1888, when Martinus Beijerink discovered that root nodule bacteria are critical to the ability of legumes to obtain nitrogen from the air. In the last two decades, much work has focused on identifying the breadth and extent of the beneficial effects of bacteria on plants with the goal of exploiting these benefits to improve plant productivity. Mutualisms between bacteria and plants can be divided into those that involve close associations between the bacteria and plant, often called symbioses, and those that involve only loose associations, often called associative interactions. In the former, the bacterial mutualist is often called a symbiont. These mutualists include some who provide a clear and direct benefit to the plant, such as the nitrogen-fixing mutualists, some who are more complex in their benefit, such as those that produce phytohormones that beneficially alter plant physiology, and some whose benefit is indirect, such as those that reduce the negative effects of pathogens.

4.1. Symbiotic N_2-fixing organisms

Biological nitrogen fixation, or the ability to convert dinitrogen to ammonia, is limited to prokaryotes but is widespread among prokaryotic genera. Among the approximately 100 nitrogen-fixing, or *diazotrophic,* genera, only a few exhibit the highly specific, intimate interactions needed to induce a new organ or organ-like structure in the host. The intimacy of such associations maximizes the transfer of fixed nitrogen to the plant, thus providing a nutrient that is often in short supply. It also provides the prokaryote with a protected environment and a supply of energy-rich compounds, which are necessary to support the high energy demands of nitrogen fixation. Symbiotic diazotrophs include legume symbionts, which are Gram$^-$ bacteria that form nodules on leguminous plants, and members of the actinomycetous genus *Frankia,* which are Gram$^+$ bacteria that form nodules on woody, dicotyledonous trees and shrubs. In contrast to these symbioses with higher plants, cyanobacteria are symbiotic diazotrophs that form mutualisms with primitive plants, described below, as well as with fungi in mutualisms called lichens; these cyanobacterial symbionts are often called *cyanobionts*. Collectively, these mutualisms result in the efficient fixation of large amounts of nitrogen and are responsible for a significant proportion of the global conversion of atmospheric dinitrogen to ammonia, nitrate and organic compounds.

4.1.1. Legume symbionts

The most well-studied plant-bacterial mutualisms are those between members of the Order Rhizobiales and members of the Leguminoseae (Fabaceae) family of plants. This plant family includes crops such as peas, beans, lentils and alfalfa, as well as trees such as locust, acacia and mimosa. All legume symbionts were originally classified into the genus *Rhizobium*, hence these symbionts are often referred to generically as rhizobia and the symbioses as *Rhizobium*-legume symbioses. Today, these symbionts are classified into several genera, with most species belonging to the genera *Rhizobium, Sinorhizobium* (*Ensifer*), *Mesorhizobium,* and *Bradyrhizobium* (Table 2). These organisms are phylogenetically clustered with non-phytosymbionts (Fig 1). Biological nitrogen fixation by legume symbionts occurs only within nodules, which are specialized outgrowths of plant tissue formed specifically in response to the bacterial symbionts. The agricultural importance of this mutualism was realized during the many centuries of crop rotation practice in which a rotation with legumes was used to rejuvenate the soil; the biological basis of this agricultural practice, however, was understood only with the recognition of the symbiosis.

Table 2. Plant-pathogenic bacteria and plant symbionts[1].

Order/Family (Fam)	**Genus**	**Species**[2]
Class: Cyanobacteria		
Subsection 4 Fam: 4.1	***Anabaena***	**spp.**
	Nostoc	**spp.**
Class: α-proteobacteria		
Order: Rhodospirillales Fam: Acetobacteraceae	*Acetobacter*	*aceti, pasteurianus*
	Gluconobacter	*oxydans*
Order: Sphingomonadales Fam: Sphingomonadaceae	*Sphingomonas*	*suberifaciens* (prev. *Rhizomonas suberifaciens*)
Order: Rhizobiales Fam: Rhizobiaceae	*Rhizobium*	***etli***[3] **(prev. *Rh. legminosarum* bv. *phaseoli* type I), *galagae* (prev. *Rh. leguminosarum*), *gallicum*[3], *giardinii*[3], *hainanense, huautlense, indigoferae, leguminosarum*[3], *lupini, mongolense, sullae, tropici* (prev. *Rh. leguminosarum* bv. *phaseoli* type II), *yanglingense***

	Agrobacterium/ Rhizobium[4]	*larrymoorei, radiobacter, rhizogenes, rubi, tumefaciens (= radiobacter), vitis*
	Sinorhizobium/ Ensifer[6]	***adhaerens, arboris, fredii***[5] **(prev. *Rh. fredii*), *kostiensis, kummerowiae, medicae, meliloti* (= *Ens. meliloti*, prev. *Rh. meliloti*), *morelense, saheli, terangae***[3]**, *xinjiangense***
Fam: Phyllobacteriaceae	*Mesorhizobium*	***amorphae, chacoense, ciceri* (prev. *Rh. ciceri*), *huakuii* (prev. *Rh. huakuii*), *loti* (prev. *Rh. loti*), *mediterraneum* (prev. *Rh. mediterraneum*), *plurifarium, tianshanense* (prev. *Rh. tianshanense*)**
	"Candidatus Liberibacter"	*asiaticus, africanus* (subsp. *africanus, capensis*)
Fam: Bradyrhizobiaceae	*Bradyrhizobium*	*betae*[7], ***canariense***[7], ***elkanii, japonicum* (prev. *Rh. japonicum*), *liaoningense, yuanmingense***
	Blastobacter	***denitrificans***
Fam: Methylobacteriaceae	*Methylobacterium*	***nodulans***[7]
Fam: Hyphomicrobiaceae	*Azorhizobium*	***caulinodans***
	Devosia	***neptuniae* (prev. *Rh. neptunii*)**
Class: β-proteobacteria		
Order: Burkholderiales Fam: Burkholderiaceae	*Burkholderia* (prev *Pseudomonas*)	*andropogonis, caryophylli, cepacia, gladioli*[8], *glumae, plantarii,* ***phymatum, tuberum***
	Ralstonia	*solanacearum* (prev. *Bu. solanacearum*), *syzgii* (prev. *Ps. syzgii*)
	Wautersia	*taiwanensis* (prev. *Ra. taiwanensis*)
Fam: Oxalobacteraceae	*Herbaspirillum*	*rubrisubalbicans* (prev. *Ps. rubrisubalbicans*)
Fam: Comamonadaceae	*Acidovorax*	*anthurii, avenae* (subsp. *avenae, citrulli, cattleyae*) (prev. *Ps. avenae* subsp. *avenae, Ps. avenae* subsp. *citrulli, Ps. cattleyae*), *konjaci* (prev. *Ps. avenae* subsp. *konjaci*), *valerianellae*
	Xylophilus	*ampelinus* (prev. *Xa. ampelinus)*
Class: γ-proteobacteria		
Order: Xanthomonadales Fam: Xanthomonadaceae	*Xanthomonas*	*albilineans, arboricola*[8,9] (= *Xa. juglandis*), *axonopodis*[8], *bromi*[9] (= *Xa. campestris* pv. *bromi*), *campestris*[8], *cassavae*[9] (prev. *Xa. campestris* pv. *cassavae*), *citri*[9]

		(= *Xa. campestris* pv. *citri* = *Xa. axonopodis* pv. *citri*), *codiaei*[9] (= *Xa. campestris* pv. *poinsettiicola*), *cucurbitae*[9] (prev. *Xa. campestris* pv. *cucurbitae*), *cynarae, fragariae, hortorum*[8,9] (= *Xa. hederae*), *hyacinthi*[9] (prev. *Xa. campestris* pv. *hyacinthi*), *melonis*[9] (= *Xa. campestris* pv. *melonis*), *oryzae*[8], *phaseoli*[9] (=*Xa. campestris* pv. *phaseoli* = *Xa. axonopodis* pv. *phaseoli*), *pisi*[9] (prev. *Xa. campestris* pv. *pisi*), *populi, sacchari*[9] (=*Xa. albilineans*), *theicola*[9] (= *Xa. campestris* pv. *theicola*), *translucens*[8] (prev. *Xa. campestris* pv. *hordei*), *vasicola*[8,9] (=*Xa. campestris* pvs. *holcicola, vasculorum*), *vesicatoria*[9] (= *Xa. campestris* pv. *vesicatoria* or *Xa. exitiosa*)
	Xylella	*fastidiosa*
Order: Pseudomonadales Fam: Pseudomonadaceae	*Pseudomonas*	*agarici, amygdali, asplenii, avellanae, beteli, cannabina, caricapapayae, cichorii, cissicola, corrugate, costantinii, flectens, fuscovaginae, marginalis*[8], *mediterranea, orrugata, palleroniana, salomonii, savastanoi*[8,9] (= *Ps. syringae* pv. *savastanoi* = *Ps. syrinage* subsp. *savastanoi*), *syringae*[8], *tolaasii, tremae, viridiflava*
Order: Enterobacteriales Fam: Enterobacteriaceae	*Brenneria* (prev. *Erwinia*)	*alni, nigrifluens, paradisiaca, quercina, rubrifaciens, salicis*
	Enterobacter	*cancerogenus* (prev. *Er. cancerogena*), *dissolvens, nimipressuralis* (prev. *Er. nimipressuralis*), *pyrinus*
	Erwinia	*amylovora, carnegieana, cypripedii* (= *Pe. cypripedii*), *mallotivora, papayae*[7], *persicina, psidii, pyrifoliae, rhapontici, tracheiphila*
	Pantoea (prev. *Erwinia*)	*agglomerans* (= *Er. herbicola* = *En. agglomerans*), *ananatis*[8], *citrea, stewartii* (subsp. *stewartii, indologenes*)
	Pectobacterium (prev. *Erwinia*)	*atrosepticum* (prev. *Er. carotovora* subsp. *atroseptica*),

		betavasculorum (prev. *Er. carotovora* subsp. *betavasculorum*), *cacticida, carotovorum* (subsp. *carotovorum, odoriferum*), *chrysanthemi* (= *Er. chrysanthemi*), *cypripedii* (= *Er. cypripedii*), *wasabiae* (prev. *Er. carotovora* subsp. *wasabiae*)
	"Candidatus Phlomobacter"	*fragariae*
	Samsonia	*erythrinae*
	Serratia	*proteamaculans* (subsp. *proteamaculans*) (prev. *Er. proteamaculans*)
Class: "Clostridia"		
Order: Clostridiales Fam: Clostridiaceae	*Clostridium*	*puniceum*
Class: Mollicutes		
Order: Entomoplasmatales Fam: Spiroplasmataceae	*Spiroplasma*	*citri, kunkelii, phoeniceum*
Order: Acholeplasmatales Fam: Acholeplasmataceae	*"Candidatus Phytoplasma"*[7]	*allocasuarinae, asteris, aurantifolia, australiense, australasia, brasiliense, castaneae, cyondontis, fraxini, japonicum, mali, oryzae, phoenicium, pini, prunorum, pyri, rhamni, spartii, trifolii, ulmi, ziziphi*
Class: "Bacilli"		
Order: Bacillales Fam: Bacillaceae	*Bacillus*	*megaterium, pumilus*
Class: Actinobacteria Subclass:Actinobacteridae		
Order: Actinomycetales Suborder: Micrococcinaea Fam: Micrococcaceae	*Arthrobacter*	*ilicis* (prev. *Corynebacterium ilicis*)[10]
Fam: Microbacteriaceae	*Clavibacter*	*michiganensis* (subsp. *insidiosus, michiganensis, nebraskensis, sepedonicus, tessellarius)* (prev. *Corynebacterium michiganense* subsps. *insidiosum, michiganense, nebraskense, sepedonicum tessellarius*)
	Curtobacterium	*flaccumfaciens*[8] (prev. *Corynebacterium flaccumfaciens* subsp. *flaccumfaciens*)
	Leifsonia (prev. *Clavibacter*)	*xyli* (subsp. *xyli, cynodontis*)
	Rathyibacter	*iranicus, rathayi,tritici*

	(prev. *Clavibacter*)	
Suborder:Corynebacterineae Fam: Nocardiaceae	*Nocardia*	*vaccinii*
	Rhodococcus	*fascians* (prev. *Co. fascians*)
Suborder: Streptomycineae Fam: Streptomycineae	*Streptomyces*	*acidiscabies, albidoflavus, aureofaciens, cacaoi* (subsp. *cacaoi, asoensis*), *candidus, caviscabies, clavifer, collinus, europaeiscabiei, fimbriatus, globisporus* (subsp. *globisporus, caucasicus), griseus* (subsp. *alpha, cretosus, griseus, solvifaciens*), *intermedius, ipomoeae, longisporus, parvulus, praecox, reticuliscabiei, rimosus* (subsp. *rimosus, paromomycinus*), *sampsonii, scabiei* (= *St. scabies*), *setonii, sparsogenes, stelliscabiei, tricolor, turgidiscabies, violaceus, wedmorensis*
Suborder: Frankineae Fam: Frankiaceae	*Frankia*	*alni*

[1] *Organisms were identified as plant pathogens (normal text) as indicated by the International Society for Plant Pathology (Young, Bull et al., 2004; Young et al., 1995) as plant symbionts (bold text) as indicated by Sawada et al. (2003).*

[2] *Prev., previous name; "=" indicates synonyms (included only homotypic synonyms and senior heterotypic synonyms); pv., pathovar; bv., biovar; subsp, subspecies.*

[3] *This species has been further divided into biovars.*

[4] *Young et al. (2001; 2003) have proposed to amalgamate the genera Rhizobium, Allorhizobium, and Agrobacterium into one genus named Rhizobium, although this emendation is not fully supported within the scientific community (Farrand et al., 2003).*

[5] *This species has been further divided into chemovars.*

[6] *Recent evidence supports the amalgamation of the genera Sinorhizobium and Ensifer into one genus, although the name of the resulting genus has been under debate (Willems et al., 2003; Young, 2003).*

[7] *These species have been proposed since the most recent release of the Bergey's Taxonomic Outline (May 2004): Bradyrhizobium betae (Rivas et al., 2004), Bradyrhizobium canariense, Methylobacterium nodulans (Jourand et al., 2004), Er. papayae (Gardan et al., 2004), and all of the "Candidatus Phytoplasma" species except ulmi (IRPCM, 2004; Lee et al., 2004; Schneider et al., 2005)*

[8] *This species has been further divided into pathovars.*

[9] *These taxonomic classifications have been subject to evaluation by Schaad et al. (2000).*

[10] *Young et al. (2004) have proposed that pathogenic strains of this species be reclassified as Curtobacterium flaccumfaciens pv. ilicis.*

Most *Rhizobium*-legume symbioses involve the development of nitrogen-fixing nodules on roots. Root nodulation involves signal exchange between the bacteria and the plant: the bacteria sense specific plant-derived signals, often flavanoids, and in response produce lipochitooligosaccharide signal molecules called Nod factors. These Nod factors induce root hair curling, localized hydrolysis of the plant cell wall within the curl, invagination of the plasma membrane, and deposition of new plant cell wall material to create an infection thread, which serves as a passageway into the plant tissue. The bacteria divide within the infection thread as it develops through the many layers of cortical cells in the root. Alternatively, some legume symbionts enter the plant through gaps in the epidermis and move through the root intercellularly, with or without the induction of an infection thread. In some host plants, such as alfalfa and pea, the bacteria induce mitotic division of the inner cortical cells and induce indeterminate nodules, which continuously elongate due to a persistent meristem at the apex. In other host plants, such as soybean, the bacteria induce mitotic division of the outer cortical cells and induce determinate nodules, which are spherical and reach a terminal size due to only transient meristemmatic activity. In two of the three legume subfamilies (Mimosoideae and Papilionoideae), bacteria move from the infection thread into the cytoplasm of the host cells by endocytosis, allowing the bacteria to become enveloped by the plant membrane. These intracellular, enveloped bacteria, called bacteroids, divide until they fill the host cell and differentiate into a form that is rich in nitrogenase, a key enzyme for nitrogen fixation. In the Caesalpinoideae legume subfamily, the bacteria do not leave the infection threads; instead, the infection threads branch until they fill the host cell. Nitrogen fixation by legume symbionts is induced only when the concentration of free oxygen in the nodule is low enough to permit activity of the oxygen-sensitive enzyme nitrogenase. The oxygen-binding plant-derived protein leghemoglobin, which is functionally related to hemoglobin, plays a key role in reducing the free oxygen levels while making oxygen available for bacterial respiration. The resemblance of nitrogen-fixing, intracellular, bacterial-derived structures to organelles such as chloroplasts have led to their designation as *symbiosomes*.

At least one legume symbiont, *Azorhizobium caulinodans,* can induce the formation of stem nodules. These have been found on the tropical host *Sesbania rostrata*. *A. caulinodans* initiates infection by entering through the gaps in the epidermis on the stem and moving through the intercellular spaces, with subsequent induction of an infection thread. Stem nodules have also been found on *Aeschynomene* species*,* and interestingly, some of these symbionts are not only diazotrophic, but also photosynthetic (Giraud & Fleischman, 2004), a relationship that could alleviate some of the energy demand by the bacteria in the nodules. Legume symbionts have been found to form nodules on a non-legume, the tropical tree *Parasponia* in the family Ulmaceae (Vessey et al., 2004). The nodules on this plant species are indeterminate and branched, similar to those of actinorhizal nodules, described below, and are induced by bacteria in the genera *Bradyrhizobium* and *Rhizobium.*

Nodulation is a strongly species-specific process, with individual bacterial strains often forming nodules only on specific plant species. In fact, species-specificity was central to the development of the taxonomy of these organisms. Whereas all legume symbionts were originally classified into the genus *Rhizobium*, the classification at the species level was based primarily on host specificity. As more isolates were examined on a wider range of legumes, it became clear that many strains can nodulate more than one host species, and distinct legume symbionts can be isolated from a single legume species, although individual nodules typically contain a single strain. Thus, host specificity gradually lost its reliability as a taxonomic criterion (Sawada et al., 2003). The application of new taxonomic criteria, including phenotypic and genotypic characteristics, chemotaxonomic markers (e.g., fatty acids) and molecular phylogenetic data (16S rRNA gene sequences and protein-coding gene sequences) along with advances in systematics, resulted in a revised classification. In the early 1990's, legume symbionts were classified into four genera: the fast-growing strains into *Rhizobium, Sinorhizobium*, and *Azorhizobium*, and the slow-growing strains into *Bradyrhizobium*. A group of fast-growing strains was subsequently recognized as a distinct taxon and the genus *Mesorhizobium* was created, with "meso" referring to its intermediate position between the other groups based on its phylogenetic position and the intermediate growth rate of its members. Strains of *Mesorhizobium* are also intermediate in having 2 rRNA operons versus 3 in the fast growers and 1-2 in *Bradyrhizobium*, and in having their nodulation and fixation genes on a megaplasmid or on the chromosome versus only on a megaplasmid like the fast growers and only on the chromosome like *Bradyrhizobium*. Today, the majority of legume symbionts are classified in the genera *Rhizobium*, *Sinorhizobium* (*Ensifer*), *Mesorhizobium*, and *Bradyrhizobium*, with host species specificity often reflected at the species level as well as at the subspecies level by divisions known as *biovars,* a term used to indicate that an organism is capable of nodulating one or more host species (Table 2). Speciation of these organisms, however, is still experiencing rapid changes as illustrated by the classification of symbiotic diazotrophs in these genera into 15 species in 1996 (Young & Haukka, 1996) but into 37 species in 2004 (Young, Park et al., 2004). Phylogenetic analyses of *B. japonicum* strains indicate that they are too diverse to be grouped into one species, so modifications to the species classification in this genus can be expected in the future. Root-nodulating diazotrophs have recently been isolated and classified as members of the genera *Blastobacter, Devosia, Methylobacterium*, and *Ochrobactrum*, all of which are also in the Rhizobiales order, as well as *Ralstonia,* which is in the Burkholderiales order (Table 2) (Rivas et al., 2004; Rivas et al., 2003; Sy et al., 2001; Trujillo et al., 2005; van Berkum & Eardly, 2002). Interestingly, tripartite mutualisms have been reported that involve mycorrhizal fungi incorporated into root nodules, or bacteria closely related to *Burkholderia* located intracellularly in mycorrhizal fungi (Bonfante, 2003).

4.1.2. Frankia

The actinomycete *Frankia*, which is a Gram$^+$ prokaryote that grows as branched hyphae, can form root nodules on over 200 species of woody, dicotyledonous trees and shrubs, such as *Alnus* (alders)*, Casuarina*, *Ceanothus* and *Myrica,* as well as on two non-woody species of *Datisca.* These plants are collectively referred to as actinorhizal plants. They are distributed throughout tropical and temperate climates, and are often the pioneer species on nitrogen-poor soils, making them useful for the reclamation of poor soils. Similar to legumes, actinorhizal plants are likely of global importance to nitrogen cycling due to their widespread occurrence.

Frankia induce root nodules on actinorhizal plants via an infection process similar to that of the legume symbionts (Vessey et al., 2004). *Frankia* can infect intracellularly by inducing root hair deformation, infection thread development, and encapsulation of hyphae by a host-derived membrane. *Frankia* can also infect intercellularly via hyphal colonization of the cortical intercellular spaces. Both infection routes ultimately lead to mitotic activation of the root pericycle cells and nodules that are similar to indeterminate legume nodules in their elongated and persistent growth; actinorhizal nodules, however, are branched. *Frankia* cells do not form bacteroids in the host cell cytoplasm, but rather differentiate the terminal regions of their hyphae into thick-walled, lipid-encapsulated vesicles. The multiple lipid layers on the vesicles collectively function as a gas-diffusion barrier, and when the oxygen concentration is sufficient low, nitrogen fixation occurs. This ability to form vesicles permits *Frankia* to fix nitrogen in a free-living state. Like most legume symbionts, *Frankia* strains induce nodules in a host-specific manner.

Compared to the *Rhizobium*-legume symbioses, relatively little is known of actinorhizal associations; this is due, at least in part, to the fact that both *Frankia* and their hosts grow relatively slowly. Furthermore, obtaining and maintaining pure culture isolates of *Frankia* is extremely difficult. *Frankia* was isolated in pure culture in 1959, with the culture subsequently lost (Pawlowski, 2002), and again in 1978 (Callaham et al., 1978), 90 years after the first isolation of *Rhizobium* from root nodules. Genetic tools that work in *Frankia* have also been difficult to develop. To date, *Frankia* strains have been isolated from 20 of the 24 genera of actinorhizal plants, but these isolates represent only a small number of the over 200 actinorhizal plant species. Although attempts to differentiate the genus into species have been hindered by insufficient numbers of isolates, a recent study has identified at least four clades within the *Frankia* phylogenetic tree (Normand et al., 1996).

4.1.3. Cyanobionts

Mutualisms between plants and cyanobacteria primarily involve diazotrophic cyanobacteria in the genus *Nostoc*. These mutualisms involve plant structures that develop in the absence of the cyanobiont, but that are changed when colonized by the cyanobiont. Features that are similar to all of these mutualisms are the presence of mucilage in the cavities that support the cyanobiont, the sole presence of the cyanobiont in these cavities, and the formation in response to the cyanobiont of long, finger-like plant cells that extend into these cavities. These extensions likely increase the surface area of the cyanobiont-host interface and thus the opportunities for nutrient exchange. The cyanobacterial association with the angio- sperm *Gunnera* is the only cyanobacterial-plant mutualism that induces the development of a symbiotic "organ", as in the nitrogen-fixing nodules.

Cyanobionts on liverworts and hornworts. Cyanobacteria form mutualisms with only two of the 330 known liverwort genera, and with four of the six genera of hornworts (Rai et al., 2000). These cyanobionts, classified as *Nostoc*, respond to plant signals by differentiating into structures called hormogonia that are specialized for taxis, and moving toward and into mucilage-filled cavities located on the ventral surface of the gametophyte. A plant signal that induces these behaviors has been isolated and named HIF, for hormogonia inducing factor. Only compatible strains are able to successfully enter these cavities, indicating host-specificity among *Nostoc* strains, and each cavity contains only one strain of *Nostoc,* like in nitrogen-fixing nodules. The infecting cyanobiont strain multiplies within the cavities and induces plant production of multicellular filaments (hairs) that extend throughout the cavity. The host eventually releases HRF, a hormogonia repressing factor, which promotes the formation of heterocysts, which are structures that are specific to nitrogen fixation. Subsequent growth of the host and the cyanobiont is synchronized, thus maintaining a similar cyanobiont:plant ratio throughout the life of the plant.

Cyanobionts on Azolla. Cyanobacterial mutualisms with the small water fern *Azolla* have attracted much agricultural interest because of their potential to enhance rice production (Vaishampayan et al., 2001). Nitrogen limitation is a major factor limiting rice productivity, and this limitation is poorly addressed by nitrogen application due, in part, to losses via NH_3 volatilization. Studies in the last few decades have shown that biofertilizers based on the *Azolla*-cyanobacterial symbiosis can significantly enhance rice cultivation. The cyanobiont, which has historically been called *Anabaena azollae*, was recently reported to be *Nostoc* (Rai et al., 2000) or a novel organism that is neither *Anabaena* or *Nostoc* (Baker et al., 2003). The infection process is similar to that described for liverworts and hornworts except that the cyanobiont is transmitted vertically (through the germ line) via the megaspore rather than being acquired by horizontal transmission from the environment (Rai et al., 2000). It enters cavities in the cotyledonary leaves via movement from an inoculum chamber during *Azolla* development, thus avoiding the need for host production of HIF and HRF signals, and like the cyanobionts on liverworts and hornworts, becomes embedded in mucilage, fixes nitrogen, and grows in relative synchrony with the host.

Cyanobionts on cycads. Cyanobacterial mutualisms with gymnosperms are slightly more sophisticated than those with the bryophytes and pteridophyte, described above, in that they involve greater differentiation of the plant tissue (Vessey et al., 2004). *Nostoc* colonizes a mucilage-filled zone, called the *cyanobacterial zone*, between the inner and outer cortical cells of specialized cycad roots. *Nostoc* colonization induces these precoralloid roots to become geotropic, lose a papillose sheath, and develop prominent lenticels, thus transforming them into coralloid, or coral-like, roots. The plant develops elongated host cells that specifically traverse the cyanobacterial zone; similar to in other cyanobacterial mutualisms, this increases the cyanobiont-host surface contact. Subsequent growth of the host and the cyanobiont is synchronized, with only one strain of *Nostoc* present in any single coralloid root.

Cyanobionts on Gunnera. Cyanobacteria can form mutualisms with only one higher plant family, the subtropical angiosperm *Gunnera* in the Gunneraceae family. In this mutualism the cyanobiont, *Nostoc,* is located intracellularly rather than extracellularly. *Nostoc* is attracted to and enters a specific gland that is present at base of each petiole along the stem; this entry may be facilitated by entrapment in mucilage that is secreted through the glands. *Nostoc* cells move through this mucilage to a cavity at the base of the gland. If the strain is compatible with the host, it enters into the thin-walled meristemmatic cells lining the cavity. This entry is mediated by localized hydrolysis of the cell wall, entry of the cyanobiont while being enveloped by the host membrane, and formation of new cell wall around the cyanobacterial cell. Subsequent differentiation and growth of the cyanobiont is accompanied by division of the infected host cells, demonstrating that like the rhizobia, cyanobacteria can stimulate mitosis in host cells.

4.2. Associative nitrogen-fixing organisms

Many diazotrophic plant-associated bacteria benefit the plant via nitrogen fixation but do not enter into host species-specific mutualisms. These organisms, as with non-diazotrophic bacteria, vary in how closely they interact with their plant host (Table 3). For example, individual diazotrophic strains may be classified anywhere along a continuum that extends from establishing intracellular, and thus highly evolved, interactions with a host to living exclusively on a plant's surface. It is tempting to speculate that increasing closeness in this association is paralleled by increasing availability of fixed nitrogen to the plant. Although such a correlation may be generally true, it has not been demonstrated and is in fact contradicted by the inability of many strains of legume symbionts and *Frankia* to effectively fix nitrogen following nodule formation. However, this spectrum of closeness is useful for discussing the range of organisms capable of *associative nitrogen fixation*, i.e., asymbiotic plant-associated nitrogen fixation. Furthermore, it is reasonable to believe that nitrogen fixed by free-living diazotrophs on plant surfaces is transferred less efficiently to the plant host and is subject to greater losses than nitrogen fixed by endophytic bacteria.

Table 3. The spectrum of diazotroph-plant interactions.

Increasing closeness in the diazotroph-plant association →	**Endosymbionts:** bacteria are located intracellularly Example: Nodule-forming bacteria on legumes and actinorhizal plants; *Nostoc* on *Gunnera*
	Ectosymbionts: bacteria are located extracellularly Example: cyanobacterial associations with primitive plants
	Endophytes: bacteria are within the host plant tissue Example: Associations between *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. in sugarcane
	Plant surface colonists: bacteria that grow only on plant surfaces Example: diazotrophic rhizosphere colonists such as *Azospirillum* and *Klebsiella*

Many important crops, such as maize, rice, wheat, and root and tuber crops, have not evolved mutualisms that involve the synthesis of specific nitrogen-fixing organs, but some, particularly grasses, often detectably benefit from the presence of diazotrophs. The extent to which associative diazotrophs transfer fixed nitrogen directly to their host versus indirectly via their death and decomposition is not clear and has been experimentally challenging to determine. The growth-enhancing effects of some of these diazotrophs is due, at least in part, to the production of phytohormones, which can not only stimulate growth directly, but also increase the efficiency with which fixed nitrogen is taken into the plant. A primary research goal of the last century has been to extend the benefit of biological nitrogen fixation to nonleguminous plants. Although research in the 1960s, 1970s and 1980s focused on rhizosphere bacteria as sources of fixed nitrogen, more recent efforts have focused on using endophytes to provide fixed nitrogen to plants, and particularly to agronomic grasses such as wheat, rice and maize. This switch in research focus was motivated, in part, by the hypothesis described above, namely that the closer the association between a diazotroph and its host, the better the transfer of fixed nitrogen and thus the greater the benefit to the host.

4.2.1. Diazotrophic endophytes

Diazotrophic endophytes that do not form nodules have been isolated primarily from grasses such as maize, rice, sorghum, sugar cane and wheat. The most well-studied diazotrophic endophytes are *Gluconacetobacter diazotrophicus* (previously *Acetobacter diazotrophicus*) and *Herbaspirillum* spp. in sugarcane, *Azoarcus* spp.

in Kallar grass, and *Alcaligenes, Azospirillum, Bacillus, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas* and *Rhizobium* in rice and maize (James, 2000). Inoculation of these bacterial species onto roots can result in colonization of the root cortex and sometimes even entry into the vascular system with subsequent translocation to the aerial regions of the plant. Populations in the intercellular spaces and xylem vessels have been found to range from 10^1 to 10^7 cells per gram of fresh weight. N-balance, ^{15}N isotope dilution and ^{15}N natural abundance studies have provided evidence that some plants can obtain at least part of their nitrogen from associative nitrogen fixation, although whether the fixed nitrogen is transferred directly to the plant or is transferred by the death and subsequent mineralization of the cells is not clear. In many cases involving inoculation of diazotrophic endophytes, nitrogen fixation activity was detected only after amendment of roots with a carbon source, suggesting that the high energy requirement of nitrogen fixation may not be met during endophytic root colonization. Recently, however, a *Klebsiella pneumoniae* strain was shown to transfer fixed nitrogen to wheat during endophytic growth in the roots without carbon amendment, and to be capable of endophytic colonization in a broad range of hosts (Iniguez et al., 2004). Other studies have shown that up to 70% of the nitrogen in sugarcane grown with *G. diazotrophicus* can originate from biological nitrogen fixation (James, 2000). Significant efforts are being made to identify endophytic diazotrophs that promote rice growth, and these have resulted in the identification of many that enhance rice growth (Reddy et al., 2002), although whether the bacteria directly transfer the fixed nitrogen to plants has not been clearly established in most cases.

4.2.2. Diazotrophic plant surface colonists

Most nitrogen-fixing prokaryotes fix nitrogen in a free-living state, presumably for their own benefit. Nitrogen fixation in the rhizosphere is likely more common than in bulk soil because the high energy demands of nitrogen fixation can be met by the carbohydrate-rich plant exudates in the rhizosphere. *Azospirillum* species were the first diazotrophs to be isolated from the rhizosphere of plants and remain the best characterized plant-surface associated diazotrophic species. Unlike some of the endophytic diazotrophs, such as *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp., which appear to be obligate endophytes, *Azospirillum* spp. are facultative endophytes, i.e., they can live either endophytically or in the rhizosphere. *Azospirillum* spp. have been the subject of extensive inoculation efforts due to their plant growth promotion activity, although it is now generally accepted that their primary benefit to plants is due to activities such as phytohormone production or altering root morphology, as discussed below, rather than nitrogen fixation. *Azotobacter* spp. have also been the subject of extensive investigations into the role of nitrogen fixation in promoting plant growth, with similar conclusions that plant growth benefits may be due, at least in part, to activities other than nitrogen fixation. In addition to *Azospirillum* and *Azotobacter,* which are obligate aerobes,