Genus

Proteobacteria/Alphaproteobacteria/Rhizobiales/Rhizobiaceae/

Rhizobium

Frank 1889, 338[AL]

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Rhi.zo'bi.um. Gr. n. *rhiza* a root; Gr. n. *bios* life; M.L. neut. n. *Rhizobium* that which lives in a root.

Rods 0.5–1.0 × 1.2–3.0 µm. Nonsporeforming. Gram negative. **Motile by 1–6 peritrichous flagella.** Fimbriae have been described on some strains. **Aerobic**, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor. Optimal temperature for growth, 25–30°C; some species can grow at temperatures >40°C. Optimal pH for growth, 6–7; range pH 4–10. Generation times of *Rhizobium* strains are 1.5–5.0 h. **Colonies** are usually white or beige, circular, convex, semi-translucent or opaque, raised and mucilaginous, **usually 2–4 mm in diameter within 3–5 days on yeast-mannitol-mineral salts agar (YMA). Growth on carbohydrate media is usually accompanied by copious amounts of extracellular polysaccharide.** Pronounced turbidity develops after 2 or 3 days in aerated or agitated broth. Chemoorganotrophic, utilizing a wide range of carbohydrates and salts of organic acids as sole carbon sources, without gas formation. Cellulose and starch are not utilized. **Produce an acidic reaction in mineral-salts medium containing mannitol or other carbohydrates.** Ammonium salts, nitrate, nitrite, and most amino acids can serve as nitrogen sources. Strains of some species will grow in a simple mineral salts medium with vitamin-free casein hydrolysate as the sole source of both carbon and nitrogen, but strains of many species require one or more growth factors such as biotin, pantothenate, or nicotinic acid. Peptone is poorly utilized. Casein, starch, chitin, and agar are not hydrolyzed. **All known *Rhizobium* species include strains which induce hypertrophisms in plants as root nodules with or without symbiotic nitrogen fixation.** Some cells of symbiotic bacterial species enter root hair cells of leguminous plants (Family Leguminosae) via invagination or by wounds ("crack entry") and elicit the production of root nodules wherein the bacteria engage as intracellular symbionts, usually fixing nitrogen. Many well-defined nodulation (*nod*) and nitrogen fixation (*nif*) genes are clustered on large plasmids or megaplasmids (pSyms). Plasmid transfer between species results in the expression and stable inheritance of the particular plant-interactive properties of the plasmid-donor species. Plant host specificity is usually

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for a few legume genera but may, in some strains, include a wide variety of legume genera and is to some extent determined by the chemical structure of the lipochito-oligosaccharide *Nod* factors produced. These chitin-like molecules induce nodule organogenesis in the absence of bacteria. **In root nodules the bacteria occur as endophytes that exhibit pleomorphic forms, termed "bacteroids", which reduce or fix gaseous atmospheric nitrogen into a combined form utilizable by the host plant.**

The mol% G+C of the DNA is: 57–66.

Type species: **Rhizobium leguminosarum** (Frank 1879) Frank 1889, 338 (*Schinzia leguminosarum* Frank 1879, 397.)

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Number of validated species: 10

Further descriptive information

Introductory Note

This treatment of the genus *Rhizobium* also includes information on the closely related genera *Agrobacterium*, and *Sinorhizobium*. The rationale for this treatment is provided under Taxonomic Comments.

Following Bradbury (1986), strains in past literature called *Agrobacterium* biovar 1 are herein referred to as *Agrobacterium tumefaciens*. *Agrobacterium* biovar 2 strains are referred to as *Agrobacterium rhizogenes*. *Agrobacterium* biovar 3 strains are referred to as *Agrobacterium vitis* (See Table 1 in the chapter on the genus *Agrobacterium*). Where necessary, the ability of pathogenic strains to cause crown gall tumors or the hairy root condition, previously attributed to strains using the names "*Agrobacterium tumefaciens*" and "*Agrobacterium rhizogenes*", is indicated by reference to the tumorigenic or rhizogenic ability, respectively, of strains in species of *Agrobacterium*. Nonpathogenic strains previously named *Agrobacterium radiobacter* are referred to either as nonpathogenic strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* or merely as nonpathogenic *Agrobacterium* if the species designation has not been identified. Ti or Ri plasmids determine the pathogenic status of strains (see below). Species comprising pathogenic or nonpathogenic strains can be reported as tumorigenic as a (Ti strain) or (Ti), as rhizogenic as a (Ri strain) or (Ri), or as nonpathogenic strains of the species where relevant.

TABLE 1. Relationships between different proposed nomenclature for the genus *Agrobacterium*

Species names based on natural classification		Species names based on pathogenicity	
Names used in this text (after Holmes and Roberts, 1981; Bradbury, 1986; Holmes, 1988)	After Keane et al. (1970); New and Kerr (1972); Kerr and Panagopoulos (1977); Panagopoulos et al. (1978)	Allen and Holding, 1974; Approved Lists (Skerman et al., 1980)	After Kersters and De Ley, 1984a
A. tumefaciens (Ti strain or Ti)	*A. radiobacter*[a] biovar tumefaciens (biotype 1)	*A. tumefaciens*	*A. tumefaciens* (biovar 1)
A. tumefaciens (Ri strain or Ri)	*A. radiobacter* biovar rhizogenes (biotype 1)	*A. rhizogenes*	*A. rhizogenes* (biovar 1)
A. tumefaciens (nonpathogenic)	*A. radiobacter* biovar radiobacter (biotype 1)	*A. radiobacter*	*A. radiobacter* (biovar 1)
A. rhizogenes (Ti strain or Ti)	*A. radiobacter* biovar tumefaciens (biotype 2)	*A. tumefaciens*	*A. tumefaciens* (biovar 2)
A. rhizogenes (Ri strain or Ri)	*A. radiobacter* biovar rhizogenes (biotype 2)	*A. rhizogenes*	*A. rhizogenes* (biovar 2)
A. rhizogenes (nonpathogenic)	*A. radiobacter* biovar radiobacter (biotype 2)	*A. radiobacter*	*A. radiobacter* (biovar 2)
A. rubi (Ti strain or Ti)[b]	*A. radiobacter* biovar tumefaciens (biotype 2)	*A. rubi*	*A. rubi*
A. vitis (Ti strain or Ti)[b]	*A. radiobacter* biovar tumefaciens (biotype 3)	*A. vitis*	*A. tumefaciens* (biovar 3)
A. vitis (nonpathogenic)	NR[c]	NR	NR

[a]Use of the species epithet *radiobacter* in place of *tumefaciens* is now not considered acceptable in terms of the Code (Sawada et al., 1993; Bouzar, 1994).
[b]Only tumorigenic (Ti) capability has been reported for this species.
[c]NR, not recorded.

Morphology

The formation of star- or rosette-shaped aggregates of cells by several *Agrobacterium* strains has been described by Beijerinck and van Delden (1902), Stapp and Knösel (1956) and Knösel (1962). Granules of poly-β-hydroxybutyrate are common in older cells, so that upon simple staining the rods appear banded. Strains of *R. leguminosarum* often contain metachromatic granules, demonstrated by staining with methylene blue, washing with dilute iodine, and staining with neutral red (Graham and Parker, 1964). Figure 1 shows a cell of *R. leguminosarum* biovar trifolii.

All species are motile by one to six flagella. For most species examined, insertion is peritrichous. Strains in species with a single flagellum (*R. galegae*, *R. mongolense*, *Sinorhizobium fredii*, *Sinorhizobium saheli*, *Sinorhizobium terangae*, and *Sinorhizobium xinjiangense*) also appear to have peritrichous organization expressed as polar or sub-polar insertion. Only *R. hainanense* is reported as being unambiguously polarly flagellated.

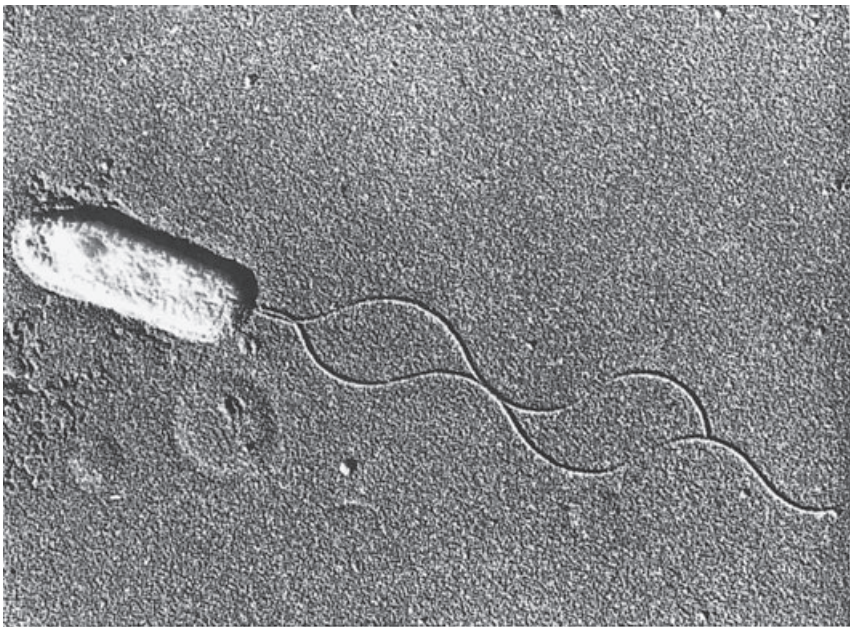
Cell wall composition

The cell wall structure of *Rhizobium* is generally similar to that of other Gram-negative bacteria. The peptidoglycan consists of glutamic acid, alanine, diaminopimelic acid, and amino sugars. In addition, leucine, phenylalanine, serine, and aspartic acid have been detected in relatively large amounts in the peptidoglycan layer of several pathogenic strains. Lipopolysaccharide (LPS) cell wall composition varies from strain to strain but consistently contains 2-keto-3-deoxyoctanoic acid (KDO), uronic acids, glucosamine, glucose, mannose, rhamnose, fucose, and galactose (Carlson, 1982). The structures of LPS from a number of species have been determined, and all of them contain the unusually long 27-hydroxyoctacosanoic acid (Jeyaretnam et al., 2002; Sharypova et al., 2003). Rhizobia also have an unusually complex composition of membrane phospholipids, among them phosphatidylcholine, and under conditions of phosphorus limitation, phospholipids can be replaced by membrane lipids that do not contain phosphorus (López-Lara et al., 2003).

Fine structure

As revealed by electron microscopy and biochemical analyses, cellulose-containing fibrils are formed by pathogenic *Agrobacterium* strains during their attachment to plant cells *in vitro*. These fibrils anchor the bacteria to the plant cell surface (Matthysse et al., 1981). Lipopolysaccharides of the outer membrane of cell envelopes play a role in the attachment of the bacteria to the wound site of the plant (Whatley et al.,

FIGURE 1. Cell of *Rhizobium leguminosarum* biovar trifolii showing two polar flagella (×14,000).



1976). Smit et al. (1989) purified an adhesin that appears to mediate the first step in attachment of nodulating *Rhizobium* and pathogenic *Agrobacterium* bacterial cells to plant root hair tips.

Colonial and cultural characteristics

On carbohydrate-containing solid media, the majority of *Rhizobium* strains produce circular, low convex to convex, mucous, glistening, opaque, white to beige-colored colonies, with an entire edge and a diameter of 2–4 mm after 5–6 days of incubation at 28°C.

Most strains grow rapidly on a mineral salts medium containing yeast extract and any one of a wide variety of carbohydrates (Vincent et al., 1979). Acid is usually produced to a moderate degree from carbohydrates (Norris, 1965).

Growth on carbohydrate-containing solid media may be opaque, clear or translucent (Figure 2) but may also exhibit small opaque areas within a clear slime. Variants that produce small colonies often fix little nitrogen symbiotically. Growth of *Agrobacterium* on nutrient agar is moderate, whereas abundant growth is obtained on media containing yeast extract and a suitable carbohydrate such as glucose, sucrose, or lactose (see Maintenance Procedures).

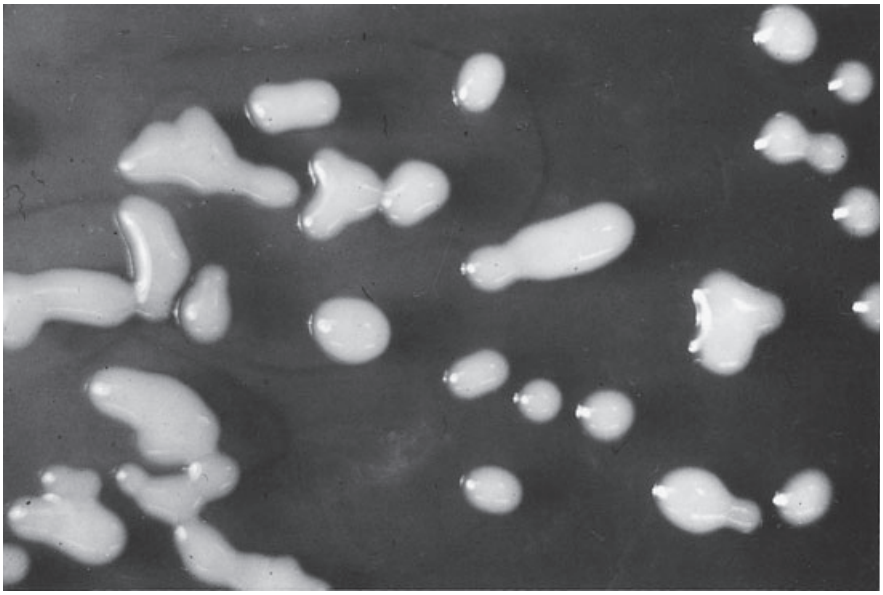
Almost all strains of *Rhizobium* species—but not *Agrobacterium* species—form only white colonies on yeast extract-mannitol-mineral-salts medium containing 0.0025% Congored.

Some strains are encapsulated. All produce abundant water-soluble extracellular polysaccharide, the principal constituent of which is acidic heteropolysaccharide (80–90%). The remaining constituents, in most strains, are neutral, unbranched, β-2-linked glucans, of which some cyclics are important for nodule development (York et al., 1980; Breedveld and Miller, 1994). Certain chromosomal genes termed *chv* or *ndv* are responsible for the production of cyclic glucans essential for either virulence or nodule development in all species of *Agrobacterium* or *Rhizobium*. All strains within a species produce the same acidic heteropolysaccharide except for *R. leguminosarum* biovar phaseoli, which possesses a unique heteropolysaccharide. Curdlan production has been reported in several isolates of *R. leguminosarum* biovar trifolii (Ghai et al., 1981). The formation of water-insoluble β-1,3-glucans has been reported in some strains of pathogenic *Agrobacterium* (Nakanishi et al., 1976). The synthesis of glycogen by *Agrobacterium tumefaciens* strain B6 is regulated at the level of ADP-glucose synthesis (Eidels et al., 1970).

Nutrition and growth conditions

The temperature range for growth, which is highly strain dependent, is 4–40°C; however, growth at 4°C is rare, and only certain species can grow at 40°C. The temperature maximum for *R. leguminosarum* is 38°C. All of the strains grow between 20°C and 28°C. *Agrobacterium rhizogenes* cannot grow above 30°C, whereas *Sinorhizobium saheli* and *Sinorhizobium*

FIGURE 2. Colonies of *Rhizobium leguminosarum* on mineral-salts mannitol agar (×1.5).



terangae can grow at 44°C (de Lajudie et al., 1994). The pH range for growth for the entire genus *Rhizobium* is 4–10.

Intermediates of the tricarboxylic acid cycle and several amino acids can be utilized as sole sources of carbon. The majority of *Agrobacterium tumefaciens* strains can grow on a minimal medium with nitrate or ammonium salts as the nitrogen source. *Agrobacterium rhizogenes* strains do not utilize nitrate unless biotin is supplied; some strains require both L-glutamic acid and biotin. Strains belonging to *Agrobacterium rubi* require L-glutamic acid and yeast extract (Starr, 1946; Lippincott and Lippincott, 1969; Keane et al., 1970).

Metabolism and metabolic pathways

The principal mechanisms of glucose catabolism in *Rhizobium* are the Entner-Doudoroff pathway and the pentose cycle (Katznelson and Zagallo, 1957; Vardanis and Hochster, 1961; Martínez-De Drets and Arias, 1972; Arthur et al., 1973, 1975; Ronson and Primrose, 1979). It is unlikely that the Embden-Meyerhof-Parnas pathway operates in *Rhizobium* spp. because activities of fructose-1,6 diphosphate aldolase and 6-phosphofructokinase are low. Polyols are substrates for an inducible dehydrogenase that converts mannitol to fructose and arabitol to xylulose (Martínez-De Drets and Arias, 1970). L-Arabinose is metabolized to α-ketoglutarate (Duncan, 1979).

The tricarboxylic acid cycle is operative, and the enzymes of the glyoxylate bypass are present (Johnson et al., 1966; Arthur et al., 1973; Chern et al., 1976a). Pyruvate carboxylase is an important anaplerotic enzyme (Chern et al., 1976b; Ronson and Primrose, 1979).

Glucuronic acid and glucaric acid are metabolized via 2-keto-3-deoxy-D-glucaric acid to α-ketoglutaric acid (Chang and Feingold, 1970). The initial step in the catabolism of L-sorbose by some *Agrobacterium tumefaciens* strains is the reduction to sorbitol, followed by oxidation of the latter compound to D-fructose (Van Keer et al., 1976). The majority of *Agrobacterium* strains characteristically oxidize a large number of carbohydrates (disaccharides, bionic acid, and several monosaccharides) to the corresponding 3-uloses (Bernaerts and De Ley, 1960a, b; Fukui et al., 1963; De Ley et al., 1966). The vigorous and unusual oxidation of lactose to 3-ketolactose is, so far, unique to *Agrobacterium tumefaciens* and is the basis of a simple and specific diagnostic test for the rapid differentiation of this species from other *Agrobacterium* and *Rhizobium* spp. that have been tested (Bernaerts and De Ley 1963; de Lajudie et al., 1994). These specific oxidations are catalyzed by an inducible hexopyranoside:cytochrome *c* oxidoreductase (D-glucoside 3-dehydrogenase), containing flavin adenine dinucleotide as cofactor (Hayano and Fukui, 1967; Van Beeumen and De Ley, 1968; Nakamura and Tyler, 1977). Although an "alpha-3"-ketoglucosidase was detected in a strain of *Rhizobium* (Hayano and Fukui, 1970; Hayano et al., 1973), 3-ketosucrose and 3-ketolactose are probably not involved as essential intermediates in the metabolism of sucrose and lactose, respectively (Kurowski and Pirt, 1971). Conditions have been worked out for increasing the yield of

3-ketoglycosides (Tyler and Nakamura, 1971; Fensom et al., 1974; Kurowski et al., 1975).

Anaerobic growth by nitrate reduction has been reported for *Sinorhizobium fredii* (Hynes et al., 1985) and *Sinorhizobium meliloti* (Daniel et al., 1982). *Sinorhizobium fredii* produces N_2O and *Sinorhizobium meliloti* produces N_2 as the end products of denitrification.

Rhizobium contains at least two soluble cytochromes *c*: a cytochrome c_{552} and a cytochrome c_{556}. Cytochrome c_{552} has been sequenced (Van Beeumen et al., 1980). It belongs to the cytochrome *c* sequence class IB (*sensu* Ambler, 1973) and, of all known procaryotic cytochromes *c*, shows the highest amino acid sequence homology with mitochondrial cytochrome *c* of tuna fish (Van Beeumen et al., 1980). Cytochrome c_{556} from *Rhizobium* belongs to the cytochrome *c* sequence class II (*sensu* Ambler, 1973), because its single heme group is bound near the C-terminus (Van Beeumen et al., 1980).

Auxotrophic mutants of *Rhizobium* are sometimes symbiotically defective and the isolation and study of such mutants has provided insights into the biochemical prerequisites of symbiosis (Kuykendall, 1981). For example, auxotrophy toward adenine, uracil, and leucine is often associated with symbiotic ineffectiveness in *Sinorhizobium meliloti* (Dénarié et al., 1976).

Fatty acids

Fatty acid profiles have been studied for some species. Using principal components analysis of whole cell fatty acid methyl esters (FAME), Jarvis et al. (1996) reported three clusters: 1) *R. leguminosarum*, *R. etli*, *R. tropici*, *Agrobacterium rhizogenes*, *Sinorhizobium fredii*, and *Sinorhizobium meliloti*; 2) *R. galegae*, *Agrobacterium tumefaciens*, *Agrobacterium rubi*, and *Agrobacterium vitis*; and 3) *R. huakuii* and *R. loti*, (now allocated to *Mesorhizobium*). Sawada et al. (1992d) could differentiate *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Agrobacterium vitis* using fatty acid profiles. *Agrobacterium tumefaciens* could be separated from *A. vitis* based on quantitative differences in $C_{16:1}$ and $C_{18:1}$ acids, and the presence of $C_{17:0\ cyclo}$ acids. Quantitative differences in $C_{19:0\ cyclo}$, and the presence of $C_{15:0\ iso\ 3OH}$ acids and the absence of $C_{18:1\ 3OH}$ acids differentiated *Agrobacterium rhizogenes* from the other two species (Sawada et al., 1992d). Tighe et al. (2000) studied 600 strains and found that the fatty acid composition of all the fast-growing species differed from that of *Bradyrhizobium* and *Mesorhizobium*, but there were no clear differences among fast-growing genera except for the relative concentration of $C_{16:0\ 3OH}$ fatty acid, which was quantitatively lower in *Sinorhizobium* than in most *Rhizobium* strains. Species-level identification was, however, possible in many cases, and thus the data of Tighe et al. (2000) is a valuable resource.

Genetics

A circular linkage map of the *R. leguminosarum* chromosome was first constructed by Beringer and Hopwood (1976). The genome of *Sinorhizobium meliloti* has also been mapped (Kondorosi et al., 1977; Meade and Singer, 1977). These two species have similar chromosomal gene arrangements (Beringer et al., 1987). *Sinorhizobium meliloti* has a chromosome size of 3.7×10^6 bp, and its sequence has been reported (Galibert et al., 2001).

There are only incomplete comparisons of *Rhizobium* spp. by DNA–DNA reassociation. DNA–DNA reassociation data are available for the following species: *R. galegae*, *R. leguminosarum*, and *R. tropici* (Martínez-Romero et al., 1991); *Sinorhizobium fredii* and *Sinorhizobium meliloti* (de Lajudie et al., 1994); and *Sinorhizobium saheli* and *Sinorhizobium terangae* (de Lajudie et al. 1994). An early study was made of *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Agrobacterium rubi* (De Ley 1972, 1974).

Easily amplified DNA regions called amplicons, apparently controlling both adaptability and biological interactions, have been documented in *Rhizobium* genomes (Palacios et al., 1998). As these become better defined through mapping and DNA sequencing, their structure can be expected to be of particular interest.

Plasmids

Plasmids and megaplasmids as large as 1600 kb can constitute as much as 50% of the total genome. Sequences of plasmids have been reported (Freiberg et al., 1997; Barnett et al., 2001; Finan et al., 2001).

Hypertrophic activity and plasmids

All species (though not all strains) of *Rhizobium* and *Agrobacterium* are associated with plant hypertrophisms and, with a few exceptions, are associated with root hypertrophisms. Hypertrophy can involve formation of either nitrogen-fixing nodules in *Rhizobium* or of oncogenous (tumorous) galls or hairy roots in *Agrobacterium*. These activities are confined to particular species. Genes (pSym, pTi, and pRi) associated with hypertrophisms are usually carried on large plasmids. These genes and the associated hypertrophying activity play a central role in the ecology of these genera. Plant transformations using the agrobacterial tumorigenic system are now routine (see reviews by Kado, 1991; Zambryski, 1992;

Hooykaas and Beijersbergen, 1994). Transformations in filamentous fungi have also been reported (De Groot et al., 1998).

Plasmid-regulated nitrogen fixation

Some members of the *Rhizobiaceae* and *Phyllobacteriaceae* are characterized by their ability to incorporate functional genetic elements as plasmids or symbiotic islands (Sullivan and Ronson 1998) which permit them to establish pathogenic (oncogenic) or symbiotic nitrogen-fixing relationships with plants. Nitrogen-fixing symbioses involving members of the *Rhizobiaceae* are restricted to plants of the family Leguminosae (van Rhijn and Vanderleyden, 1995), with one exception. *Rhizobium* cells contain as many as 10 naturally occurring plasmids ranging in size from less than 100 kb to megaplasmids of more than 1000 kb. In some instances, the combined length of plasmids and megaplasmids approximates that of the chromosome, meaning that up to 50% of the *Rhizobium* genome is not in the chromosome. Both *nod* and *nif* genes controlling nodulating and nitrogen-fixing ability have always been found clustered together on one or more *Rhizobium* plasmids or megaplasmids called pSym. Numerous genetics studies have delineated *Rhizobium* genes essential for legume nodulation and symbiotic nitrogen fixation (Rossen et al., 1984; Török et al., 1984; Egelhoff and Long, 1985; Egelhoff et al., 1985; Jacobs et al., 1985; Debellé et al., 1986; Evans and Downie, 1986; Göttfert et al., 1986; Horvath et al., 1986; Rostas et al., 1986; Shearman et al., 1986; Aguilar et al., 1987; Fisher et al., 1987; Honma and Ausubel, 1987; Cremers et al., 1988; Davis et al., 1988; Surin and Downie, 1988; Cervantes et al., 1989; De Maagd et al., 1989; Schwedock and Long 1989, 1994; Barnett and Long, 1990; Economou et al., 1990; Honma et al., 1990; Surin et al., 1990; Baev et al., 1991, 1992; Kondorosi et al., 1991a, b; Rushing et al., 1991; Baev and Kondorosi, 1992). The nodulation-controlling genes are organized into several coordinately regulated operons. For example, the common *nodABC* operon is present in all legume symbiotic strains and can complement strains in different genera. For *R. etli*, *nodA* is separated from *nodBC* (Vázquez et al., 1991; Vázquez et al., 1993), and for *Mesorhizobium loti*, *nodB* is independent of *nodAC* (Scott et al., 1996). Others may be present in different species as allelic variants, such as *nodEF*, and these are host-specific and hence not interchangeable. Such *nod* or *hsn* genes are sometimes present only in certain strains.

pSyms are not essential for survival of the *Rhizobium* strains in soil. Non-nodulating soil bacteria identified as *Rhizobium* species have been isolated which can only form nodules after transconjugation with related symbiotic strains. Transfer of symbiotic plasmids among *Rhizobium* species has been reported under laboratory conditions and has been demonstrated by sequence comparison of natural isolates. Martínez et al. (1987) demonstrated nitrogen-fixing nodules formed by *Agrobacterium tumefaciens* carrying a conjugally transferred pSym from *R. tropici* (as *R. phaseoli* type II). Similar results were obtained for transconjugants of *Agrobacterium tumefaciens* containing pSym from *Rhizobium* strains that nodulate *Phaseolus vulgaris* (Brom et al., 1988). A pSym of *R. leguminosarum* biovar trifolii has been introduced into 15 non-nodulating bacterial isolates identified as *M. loti*, *R. leguminosarum*, *R. tropici*, *Sinorhizobium meliloti*, and four isolates related to *R. leguminosarum* (Sivakumaran et al., 1997). By comparison of *nifH* sequence type to 16S rRNA gene sequence type, Haukka et al. (1998) demonstrated that similar symbiotic genes could be found in different 16S rRNA gene backgrounds, indicating horizontal transfer across species boundaries.

There is considerable variation in the nodulating and nitrogen-fixing capacity of individual strains. In *Rhizobium* strains with the capacity for symbiotic activity, most if not all genes that specify and regulate nodulating and nitrogen-fixing abilities are carried on one or more plasmids. In this respect, members of the genus differ from *Bradyrhizobium* (see the chapter on the genus *Bradyrhizobium*), in which all symbiosis-controlling genes have been shown to be carried on the chromosome, and *Mesorhizobium* (see the chapter on the genus *Mesorhizobium*), in which many strains have symbiosis-controlling genes located on the chromosome. The systematics of these plasmid-determined symbiotic associations is reviewed in Young and Johnston (1989).

The expression of nodulation genes is controlled by the presence of flavonoids excreted by the host plant. For, example, the regulatory *nodD* gene controls *nodABC* expression. Flavonoids produced by various legumes seem to interact specifically with particular NodD proteins, which vary in structure according to *Rhizobium* species, as do the Nod Factors (NF). Compatibility between flavonoid and NodD protein is thought to be a major factor in host specificity. (For detailed reviews of nodulation and nitrogen fixation genetics and biochemistry, see Schultze et al., 1994; van Rhijn and Vanderleyden, 1995; Dénarié et al., 1996.) Another regulation gene, *nolR*, has been reported to be common in symbiotic species of *Rhizobium* and *Sinorhizobium*. This gene was not found in species of *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, or *Agrobacterium* (Kiss et al., 1998). This gene repressed the expression of both *nodABCIJ* and *nodD* genes, resulting in decreased Nod factor production.

Recently, a novel family of *nod* gene inducers, aldonic acids, was reported for *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Bradyrhizobium* (as *B. lupini*) strains (Gagnon and Ibrahim 1998).

Root nodules induced by some strains of symbiotic *Rhizobium* contain substances called rhizopines (Murphy et al., 1987, 1995), analogous to the opines found in pathogenic *Agrobacterium* strains (see later discussion). Those described so far are substituted scyllo-inosamines. Both synthetic and catabolic genes are located on pSym. Genes involved in synthesis are active in the symbiotic bacteroid state and catabolic genes are active in the free-living cells (Murphy et al., 1988; Dessaux et al., 1998). The likely ecological benefit of rhizopine activity for producing strains has been reported (Gordon et al., 1996). Rhizopine production has been reported for *R. leguminosarum* biovar viciae and *Sinorhizobium meliloti*. No rhizopine production was detected from isolates of *R. etli*, *R. tropici*, or *R. leguminosarum* biovar trifolii and biovar phaseoli (Wexler et al., 1995). Rhizopine synthesis (*mos*) and catabolism (*moc*) genes from *R. leguminosarum* and *Sinorhizobium meliloti* have been sequenced (Wexler et al., 1996b). Further study showed that some non-symbiotic soil bacteria, including *Arthrobacter*, *Aeromonas*, *Alcaligenes*, and *Pseudomonas*, could catabolize rhizopine. No DNA sequences homologous to *nodB* and *nodC* and no effective nodulation on *Medicago sativa* were detected in two strains that were related to *Sinorhizobium meliloti* based on partial 16S rDNA sequence analysis (Gardener and Bruijn, 1998); this result indicated that the gene(s) related to rhizopine metabolism may be carried on the chromosome or on plasmids other than pSym.

Two megaplasmids in *Sinorhizobium meliloti* are involved in the effective nodulation of alfalfa (Hynes et al., 1986). In *R. leguminosarum* (Hynes and McGregor, 1990), as in *R. etli* CFN42, plasmids other than the pSym (*nod-nif* plasmid) are required for an effective symbiosis and may carry *lps* genes (García-de los Santos and Brom, 1997; Vinuesa et al., 1999).

Plant specificity in nitrogen fixation

The legume-nodulating ability of *Rhizobium* species appears to be specific to a few plant species or genera. An exception is *Sinorhizobium* sp. strain NGR234, which nodulates 112 legume genera (Pueppke and Broughton, 1999). In such instances, nodulation can occur without nitrogen fixation. Since Lerouge et al. (1990) established the chemical structure of the nodule-inducing compound produced by *Sinorhizobium meliloti*, a general hypothesis has been proposed: host specificity in all legume microsymbionts is related to the chemical structure of specific lipochitooligosaccharide Nod factors (Dénarié et al., 1992, 1996). Nodulation factors of strain NGR234 include variants of Nod factors (Price et al., 1992). Structures of Nod factors produced by several species have been described (Poupot et al., 1993, 1995; Lorquin et al., 1997; Yang et al., 1999; Snoeck et al., 2001; Pacios-Bras et al., 2002).

Plasmid-mediated plant-pathogenic (**oncogenic**) activity in *Agrobacterium* spp

The early literature on plasmid-mediated plant pathogenic (oncogenic) activity was reviewed by Nester et al. (1984). A recent review is given by Binns and Costantino (1998). Oncogenic (tumorigenic or rhizogenic [hairy root]) activity in the four plant-pathogenic *Agrobacterium* species is mediated by genes that are largely or wholly borne on one or more large (>150 kb) plasmids. Tumorigenic activity is conferred by Ti plasmids and rhizogenic activity is conferred by Ri plasmids. Tumorigenic genes on the Ti plasmid comprise (a) T-DNA genes, and (b) virulence (*vir*) genes. Wounding of susceptible plant tissue activates *vir* genes that facilitate the transfer of a component of the Ti (or Ri) plasmid, the T-DNA (8–22 kb). The T-DNA fragment is integrated into the plant nucleus apparently at random (Chyi et al., 1986), in one or more copies (Chilton et al., 1980; Lemmers et al., 1980; Willmitzer et al., 1980; Zambryski et al., 1980). T-DNA carries all necessary genes for tumor growth, the most important for tumorigenesis being those associated with auxin and cytokinin synthesis, which are expressed in the plant.

Comparative analysis indicates a correlation between sequence structure of the 16S–23S rRNA intergenic spacer region and the type of Ti plasmid (nopaline, vitopine, or octopine/cucumopine) present in strains of *Agrobacterium vitis* (Otten et al., 1996).

Another large Ri plasmid is involved in the hairy root disease of plants caused by rhizogenic *Agrobacterium* strains (Moore et al., 1979; White and Nester, 1980a). Little overall sequence similarity to other Ti plasmids has been detected (White and Nester, 1980b). There is one small region of conserved similarity between the Ri plasmid and an octopine Ti plasmid (pTi-B6806), but the former shows no similarity to the T-DNA region of the latter plasmid. The Ri plasmid is compatible with other Ti plasmids and thus represents a new incompatibility class of plasmids (White and Nester, 1980b).

Large plasmids that are not involved in pathogenic activity have been found in nonpathogenic strains of *Agrobacterium*

tumefaciens and *Agrobacterium rhizogenes* (Merlo and Nester, 1977; Sheikholeslam et al., 1979). In addition, large plasmids have been discovered in addition to the Ti plasmid in several tumorigenic *Agrobacterium* strains. Some strains possess an additional 2.1×10^6 base-pair linear chromosome in addition to two or more very large plasmids (Allardet-Servent et al., 1993). The sequence of the *A. tumefaciens* C58 genome was reported (Goodner et al., 2001).

Opines are unusual amino acid derivatives produced in tumor or hairy root tissues induced by pathogenic strains of *Agrobacterium* species. Opines are synthesized from common plant compounds but are not utilized by plants or by most microorganisms. They are utilized by *Agrobacterium* strains that carry the specific opine-inducing plasmids associated with oncogenicity. The opine concept as originally proposed was that specific genes associated with T-DNA permitted the synthesis of opines from plant photosynthetic products in a form whose availability as nutrients was restricted to tumorigenic strains bearing the relevant plasmid (Schell et al., 1979). At least eleven opines have been identified: octopine, lysopine, nopaline, succinamopine, leucinopine, cucumopine, heliopine, chrysopine, mikimopine, agropine, and agrocinopines (Chang et al., 1989; Dessaux et al., 1992; Chilton et al., 1995). In addition, imino acids (Moore et al., 1997), mannopine (Petit et al., 1983), and vitopine (Szegedi et al., 1988) have been reported. It is unlikely that this list is exhaustive (Moore et al., 1997). Opine catabolism by pathogenic *Agrobacterium* strains is mediated by genes, located on one or more plasmids including the Ti plasmid, that are not transferred to the plant cell nucleus (Montoya et al., 1977). Pathogenic strains can utilize more than one opine (Moore et al., 1997). Some opines can also induce conjugal transfer of the Ti plasmid to nontumorigenic strains and may contribute to the dissemination of the infectious plasmid (Gelvin 1992; Guyon et al., 1993). Analysis of the distribution of Ti plasmids in terms of their opine genes showed that the ecology of plasmid-bearing strains is highly complex (Moore et al., 1997). In only a few samples could field outbreaks of crown gall be traced to a clonal origin of infection. In most collections, field tumors were induced by Ti plasmids of more than one opine type. Field tumors of some hosts yielded no detectable opines, even though opine-utilizing bacteria were present. Bacterial isolates from other hosts (plum and cherry) showed the best correspondence between the opine in tumors (nopaline) and the presence of bacteria that catabolized that opine. However, several unusual opine catabolic combinations were identified, including isolates that catabolized a variety of opines but were nonpathogenic. There are indications of some specificity between pathogenic *Agrobacterium* species, opine type, and host plant (Lopez et al., 1988; Sawada et al., 1992a). The opine concept (Schell et al., 1979) assumed specific utilization of opines by tumorigenic *Agrobacterium* strains bearing relevant plasmids, and that these compounds could not be utilized by other soil organisms. Since the original proposal, however, it has become clear that a wide range of soil organisms have the capacity to metabolize opines. These include *Pseudomonas* species (Beaulieu et al., 1983; Tremblay et al., 1987b) and Gram-positive coryneform bacteria (Tremblay et al., 1987a). These data collectively suggest that mechanisms explaining the involvement of opines may be more complex than the original model.

At present, oncogenic activity is associated with *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, and *Agrobacterium vitis*. There may be several additional pathogenic bacterial populations that also merit classification as species. Bouzar et al. (1995) records a pathogen associated with aerial infections of *Ficus benjamina*, and Sawada and Ieki (1992b) report phenotypically distinct strains isolated from affected plants.

Pathogenic host range and pathogenicity

The host range of tumorigenic *Agrobacterium* strains is reported to be very wide. De Cleene and De Ley (1976) described at least 640 plant species belonging to 331 genera in 93 families of dicotyledon and gymnosperm plants as susceptible to transformation by *Agrobacterium* (Ti strains) (such as *Agrobacterium tumefaciens*). De Cleene and De Ley (1981) reported 37 plant species belonging to 30 genera in 15 families of dicotyledonous plants as susceptible to transformation by *Agrobacterium* (Ri strains) (such as *Agrobacterium rhizogenes*). None of the 250 monocotyledonous species investigated was susceptible to the disease, except some members of the orders Liliales and Arales. Bradbury (1986) listed almost 400 plant species affected by tumorigenic strains and over 50 plant species affected by rhizogenic strains.

The inference that crown gall- and hairy-root-inducing strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have wide host ranges is based on reports of the many hosts from which the pathogens have been isolated and on the many reports of tumor induction in experimental inoculations. However, there have also been indications of host specificity within some populations (Panagopoulos and Psallidas, 1973; Anderson and Moore 1979; Moore and Cooksey 1981; Paulus et al., 1991a, b; Palumbo et al., 1998). In some instances, strains are pathogenic to a relatively narrow range of host plants (Unger et al., 1985). Furthermore, although crown gall has been reported on some host species in some

countries, it is not necessarily found in these same hosts in other countries where crown gall is known. The family of Ti plasmids may have restricted host ranges in varying degrees and chromosomal background may affect specificity. It seems clear that some strains naturally infect host plants from several unrelated genera whereas others are more specific (Paulus et al., 1991b). The nature of specificity, whether it is a function of the bacterial strain or of the plasmid (Loper and Kado 1979; Close et al., 1985; D'Souza et al., 1993), has not been generally confirmed, although modifications to the Ti-plasmid have been implicated (Paulus et al., 1991a). There is no indication of host range specificity as it occurs in the pathogenic species and pathovars of *Pseudomonas* or *Xanthomonas*. Although *Agrobacterium rubi* is identified as a species isolated from galls on the canes of *Rubus* spp., the specificity of this pathogen to *Rubus* is in doubt because it has been shown to have a wide host range (Sawada et al., 1992a). *Agrobacterium vitis* is found as the predominant tumorigenic species specific to *Vitis* spp. (Thies et al., 1991), and *Agrobacterium vitis* strains have occasionally been isolated from other hosts, such as *Actinidia* (Sawada and Ieki 1992a). *Agrobacterium vitis* appears to be unique among pathogenic *Agrobacterium* species in being associated with a root decay symptom (Burr et al., 1987).

Upon infection of wounded plant tissues, tumorigenic *Agrobacterium* strains can transform plant cells into autonomously proliferating cells. In nature, the swellings mostly occur at the transition zone between the stem and the root system of the host plant, hence the name "crown gall disease." Small spherical growths or elongated ridges can occur on the stems of *Rubus* spp. such as raspberry and bramble bushes. Some oncogenic strains cause hairy root on susceptible plants (such as apple trees and roses) but cause crown gall on other plants. On some plants (e.g., *Kalanchoë*), oncogenic strains of *Agrobacterium* spp. can induce the formation of teratomata, characterized by the development of aberrant shoots, leaves, or roots developing from the tumor tissue. The type of disease produced (differentiated or undifferentiated tumors) is probably determined by both the bacterial Ti plasmid and the host plant (Gresshoff et al., 1979).

A prerequisite for tumorigenesis is the wounding of the host. Infection can occur during various stages of the life of a plant via wounds caused by growth, germination (e.g., peaches and almond), subterranean insects, or mechanical injuries (e.g., pruning, grafting, and replanting of trees in nurseries).

Tumorigenic and rhizogenic activity was initiated by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* at 20°C and 27°C, respectively. The latter temperature, however, was not conducive to tumorigenesis by *Agrobacterium vitis*. Temperature effects were mediated by the choice of host plant (Charest and Dijon, 1985).

Crown gall disease seldom kills plants, but growth is often impaired and stunted. Significant damage and economic loss can occur on stone fruit and grape (De Cleene, 1979).

Plasmid exchange between *Rhizobium* and *Agrobacterium* species

Intergeneric transmissibility of Ti and nodulating plasmids has been demonstrated from nodulating *Rhizobium* spp. to tumorigenic *Agrobacterium* spp. (Martínez et al., 1987; Brom et al., 1988; Abe et al., 1998), and from tumorigenic *Agrobacterium* spp. to nodulating *Rhizobium* spp. (Hooykaas et al., 1977) and to *Phyllobacterium myrsinacearum* (van Veen et al., 1988); this supports a close relationship for these genera and points to promiscuous plasmid exchange between taxa. Novikova and Safronova (1992) reported transconjugants of *Agrobacterium tumefaciens* harboring the pSym genes of *R. galegae* that formed an effective symbiosis with *Medicago sativa*. The finding by Nesme et al. (1987) that crown gall that occurred in a poplar nursery was caused by naturally occurring resident mixed populations of both *Agrobacterium tumefaciens* (Ti strains) and *Agrobacterium rhizogenes* (Ti strains) supports the idea that Ti plasmids may be promiscuous in the resident tumorigenic *Agrobacterium* species. Plasmid homologies do not correlate with any numerical classification of pathogenic *Agrobacterium* spp. (Currier and Nester, 1976), hence it is generally assumed that plasmid-borne Ti and Ri genes are readily transmitted within and between strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

Although plasmids conferring pathogenicity and nodulation are transmissible between pathogenic and nodulating genera in the laboratory, in nature these characteristics appear to be specific to the particular species. The relative specificity between particular nodulating *Rhizobium* spp. and their legume symbionts, and the pathogenic specificity indicated for *Agrobacterium vitis* and, perhaps, *Agrobacterium rubi* suggests that some plasmid incompatibilities exist, causing a restriction on transmission or subsequent gene expression. Furthermore, Bouzar et al. (1993) and Otten et al. (1996) demonstrated a correlation between the form of resident plasmids and host chromosome, suggesting possible restrictions on exchange in nature.

Agrocin and trifolitoxin activity

Agrocin 84 from the nonpathogenic *Agrobacterium rhizogenes* strain 84 (ICMP 3379; NCPPB 2407) (New and Kerr, 1972; Kerr and Htay, 1974) is plasmid-encoded (Ellis et al., 1979). It is a toxic analog of an adenine nucleotide (Roberts et al., 1977) and selectively inhibits pathogenic *Agrobacterium* strains harboring a nopaline plasmid. It is effective against *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* but not *Agrobacterium vitis* or *Agrobacterium rubi* (Ma et al., 1985; van Zyl et al., 1986; Psallidas 1988; Sawada et al., 1992a). Sensitivity towards agrocin 84 is determined by the Ti plasmid. Dipping seeds, roots, or wounded plant surfaces in suspensions of strain 84 has been used with success worldwide for the biological control of crown gall disease (Moore and Warren, 1979; Kerr, 1980). Strain 84 is available in commercial preparations as a biological control agent and has found wide application. *Agrobacterium vitis* strains are insensitive to agrocin 84. Other agrocin-producing strains, effective against *Agrobacterium vitis*, have been isolated (Staphorst et al., 1985; Chen and Xiang 1986; Webster et al., 1986; Webster and Thompson 1988; Xie et al., 1993).

Trifolitoxin (TFX) is a post-translationally modified peptide antibiotic produced by *R. leguminosarum* biovar trifolii T24 (Breil et al., 1993). TFX is toxic to non-producing strains within a distinct taxonomic group of the *Alphaproteobacteria*, and it appears to give an ecological advantage for nodulation by the producing strain. Eight genes have been identified for the production of this toxin (Triplett et al., 1994; Breil et al., 1996).

Bacteriophages

Lysogeny for either active plaque-forming or defective bacteriophages is widespread in tumorigenic *Agrobacterium* spp. Morphological, biological, and physicochemical properties and genetic relationships of several of the isolated phages or phage-like particles have been determined (Beardsley, 1955; Zimmerer et al., 1966; Stonier et al., 1967; De Ley et al., 1972; Manasse et al., 1972; Vervliet et al., 1975). Virulent bacteriophages of *Rhizobium* were the subject of numerous studies published prior to 1950 (Allen and Allen, 1950), but *Agrobacterium* phages, although not as extensively studied in earlier times, have more recently been isolated from sewage and soil (Roslycky et al., 1963; Boyd et al., 1970a, b).

The range of hosts susceptible to a particular *Rhizobium* bacteriophage is highly variable. In some instances, it is limited to relatively few strains within a single host species; in others, it may cross taxonomic boundaries. Cross-infection studies of *Sinorhizobium meliloti* and *Sinorhizobium fredii* (Hashem et al., 1996) indicate that bacteriophage lysis is not usually sufficiently specific to identify species or individual strains. Some strains of *Rhizobium* are lysogenic. Bacteriocins have been reported (Roslycky, 1967; Venter et al., 2001), as well as a parasitic *Bdellovibrio*.

Antigenic structure

Early serological studies indicated that strains belonging to *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* could be distinguished from each other by serological reactions (Keane et al., 1970; Lopez, 1978). Alarcón et al. (1987) and Sawada et al. (1992c) found serological heterogeneity between these species and with *Agrobacterium vitis*. *Rhizobium* spp. show extensive cross-reaction with *Agrobacterium* strains (Graham, 1971).

Most serological reactions (agglutination, gel diffusion, precipitation, and fluorescent antibody) show strain specificity and have traditionally been of great value in identifying particular strains of *Rhizobium* in nodules of field plants or in laboratory investigations. The agglutination reaction, using crushed nodule extracts, is the most widely used for field work because of its simplicity, although it is complicated by cross-reactions and autoagglutination. Surface antigens, although useful for strain recognition, are limited in their usefulness for species identification. Early work, such as that of Vincent and Humphrey (1970), who reported on the antigen structure in the "biovars" of *R. leguminosarum*, should now be reinterpreted in the light of modern taxonomic revisions of the species. Sawada et al. (1992b) differentiated several serogroups in *Agrobacterium vitis* using a slide agglutination test.

Ecology

Rhizobium occurs worldwide in soils and especially in the rhizosphere of plants. As many as 10^6–10^7 cells/g soil of symbiotic *Rhizobium* have been reported. *Rhizobium* strains capable of degrading 2-sulfonato-fatty-acid-methyl-esters (Masuda et al., 1995) and *Agrobacterium* strains capable of utilizing phthalate (Nomura et al., 1989) were reported as common soil inhabitants in contaminated soils. The identification of these strains as authentic rhizobia and agrobacteria needs confirmation.

Natural interactions between *Rhizobium* strains within the rhizosphere are complex, as indicated by the extent to which different *Rhizobium* populations are shown to compete in the infection processes. Agrocin-producing strains have a proven role in competing with tumorigenic *Agrobacterium* strains

to inhibit infection. A similar competitive process occurs when attempts are made to nodulate seedling legumes with effective *Rhizobium* strains. Naturally occurring strains that are nonefficient in nitogen fixation can be more effective in infecting and nodulating plants, thereby limiting plant growth (Triplett and Sadowsky, 1992). Strains of *Rhizobium* that are non-nodulating and occurring naturally in soils have been well documented (Soberón-Chávez and Nájera, 1989; Segovia et al., 1991). Symbiotic *Rhizobium leguminosarum* biovar trifolii strains have been reported as natural endophytes in the roots of rice (Yanni et al., 1997), and *R. etli* strains, as endophytes of maize (Gutiérrez-Zamora and Martínez-Romero, 2001). Plant-pathogenic *Agrobacterium* species have also been isolated from a crown gall tumor on alfalfa (Palumbo et al., 1998).

Although bacteria within the genus *Rhizobium* have been shown to have a worldwide distribution, unique species may be isolated from limited geographic regions, normally related to the distribution of their hosts (Martínez-Romero and Caballero-Mellado, 1996). *Rhizobium* species may have been spread internationally with inoculated legume plants, with seed (Pérez-Ramírez et al., 1998), or soil. Caballero-Mellado and Martínez-Romero (1999) reported that soil fertilization limited the genetic diversity of *Rhizobium* in bean nodules. Vance (1998) reviewed agronomic aspects of commercial inoculants that are used to enhance legume crop cultivation.

Agrobacterium strains have also been reported in a variety of human clinical specimens (CDC group Vd-3) (Lautrop, 1967; Riley and Weaver, 1977; Gilardi, 1978a; Rubin et al., 1980). They are usually 3-ketolactose-positive and nonpathogenic to tomato. It is believed that these clinical isolates occur either as incidental inhabitants in the patient or as contaminants introduced during sample manipulation. The authenticity of these strains as true *Agrobacterium* spp. needs to be confirmed.

Antibiotic sensitivity

Rhizobium strains are resistant to a variable spectrum of antibiotics (Davis, 1962). Most are susceptible to tetracycline. Although there is wide strain-to-strain variation in resistance, *Rhizobium* strains are intrinsically more sensitive than *Bradyrhizobium* to tetracycline, penicillin G, viomycin, vancomycin, and streptomycin. Streptomycin-resistant mutants, which are usually effective as nodulating strains, are important in ecological field studies on strain competition. In general, wildtype *Agrobacterium* tumorigenic species have been reported to be sensitive to chlorotetracycline, gentamicin, neomycin, novobiocin, oxytetracycline, and tetracycline (Kersters et al., 1973) but are commonly resistant to nalidixic acid. Growth is inhibited by low concentrations (3–780 µg/ml of medium) of metacycline, doxycycline, sigmamycin (tetracycline + oleandomycin), and triacetyloleandomycin (Goedert, 1973).

Enrichment and isolation procedures

Although *Rhizobium* strains are common soil inhabitants, they are best isolated from freshly excised legume root nodules. Identification is relatively easy if strains are isolated from host plant nodules. Isolation is difficult if strains are isolated directly from the soil or if strains are non-infective, unless they have unique genetic markers. Isolation of symbiotic species from soil generally requires the use of trap hosts, which are leguminous plants grown in the soil and from which nodules are selected for subsequent isolation of rhizobia. Nodules collected in the field can be temporarily stored in small vials containing silica gel held under a cotton plug.

In order to isolate symbionts, healthy root nodules—with a small portion of root attached if they are very small (<1.0 mm)—are surface sterilized by exposure to commercial 3% H_2O_2 solution or 5% commercial hypochlorite (3% available chlorine) solution for 5–60 min depending on their size. This treatment is followed by a wash in sterile water. The nodules are crushed in a small drop of sterile 0.05% peptone or 0.1M phosphate and a loopful of the suspension is streaked onto surface-dried plates of yeast extract-mannitol agar (YMA)[1] prepared without $CaCO_3$. Alternatively, a small loopful of the crushed nodule suspension can be streaked onto successive plates of agar medium. Large nodules can be sliced with a sterile scalpel blade and portions of the interior removed with a needle. Bacteria can readily be isolated from young galls and nodules on different parts of plants. Isolation from older hypertrophying tissue is more difficult. Incubation is at 28°C for 3 or more days. Well-isolated, white, mucoid, glistening, hemispheric colonies are restreaked onto fresh plates for subsequent confirmation, which requires reinfection of the original host under careful aseptic conditions where uninoculated controls are devoid of nodules. If heavy fungal contamination is expected, the agar medium used for initial isolation should contain 0.002% actidione. *Rhizobium* strains grow poorly on 0.04% peptone/1% glucose mineral salts agar and show little pH change. This medium can serve as a useful contamination check: colony formation in 2 days at 28°C and a marked pH change are not characteristic of *Rhizobium*.

Maintenance procedures

YMA slant cultures in sealed containers can be stored for 2–3 years or longer at 2°C. Cultures usually survive for around 2 months at 15°C. Long-term storage at −20°C or −80°C is recommended: turbid suspensions from fresh broth cultures are mixed with equal volumes of sterile 80% glycerol in water and allowed to stand for 1 hr at room temperature before storing in small aliquots in the freezer. Individual aliquots are thawed as required. Norris (1963) described a preservation method using small porcelain beads which, after inoculation and drying over silica gel, can be used individually to inoculate YMB for subsequent recovery of the bacteria.

Stock cultures of pathogenic species may be routinely maintained on agar slants in screw-capped vials at 4°C for 2 months on YMA or on either of the following media (in g/l of tap water): (a) glucose, 20; yeast extract, 10; $CaCO_3$, 20; and agar, 20; or (b) glucose, 10; yeast extract, 10; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.25; and agar, 20.

Lyophilized cultures stored at 4°C remain viable for at least 25 years.

Procedures for testing special characters

Carbohydrate and organic acid utilization have been determined using inoculated plates of the medium of Elkan and Kwik (1968). On the dried surface of these plates are placed absorbent paper discs previously saturated with a 10% solution of the organic compound and slowly dried. During incubation at 28°C the plates are examined daily using indirect lighting against a black, non-reflecting background.

API Biotype galleries (BioMerieux, La Balme-les-Grottes, France) and similar standardized systems to test for the utilization of standard ranges of substrates are increasingly used to obtain reproducible biochemical data.

Details on the methods used to assess nodulation response under greenhouse or growth room conditions are given by Vincent (1970), and Somasegaran and Hoben (1994) have produced an excellent methods book for the novice researcher of symbiotic species. Moore et al. (1988) have provided useful advice for performing inoculation tests for pathogenicity studies and summarize recipes for diagnostic media and tests.

Sawada et al. (1995) have reported a method for detecting the presence of Ti and Ri plasmids by specific amplification of components using the polymerase chain reaction.

Differentiation of the genus *Rhizobium* from other genera

Members of *Rhizobium* are distinguished from those in the related genera *Mesorhizobium* and *Phyllobacterium* by differences in growth rate, fatty acid profiles, and 16S rDNA sequence. Members of *Rhizobium* are not distinguished from those in the related genera *Allorhizobium* or *Sinorhizobium* by any phenotypic characters except those that form the individual species circumscriptions. The genus *Agrobacterium* is distinguished from the genera containing nitrogen-fixing species, including *Rhizobium*, only because its members have oncogenic capabilities. *Rhizobium* is distinguished from *Sinorhizobium* not only based on differences in their 16S rDNA sequences but also on the basis of other gene sequences (Gaunt et al., 2001) and by *nolR* gene hybridization (Toledo et al., 2003).

The features that differentiate nodulating strains of *Rhizobium* species from morphologically and physiologically similar organisms are given in Tables 2 and 3. Strains of non-pathogenic agrobacteria and non-nodulating rhizobia can be isolated from soils and are difficult to allocate to genera or species based on characteristics reported in Table 2. It is necessary to resort to specific molecular probes for reliable identification.

Taxonomic comments

Overviews of the relationships of bacterial nitrogen-fixing genera are given in Young (1992, 1994), Martínez-Romero (1994), Lindström et al. (1995, 1998), Martínez-Romero and Caballero-Mellado (1996), and Young and Haukka (1996).

At that time of publication of the first edition of *Bergey's Manual of Systematic Bacteriology*, the family *Rhizobiaceae* comprised *Rhizobium* (Jordan, 1984a), *Bradyrhizobium* (Jordan, 1984a), *Phyllobacterium* (Knösel, 1984a), and *Agrobacterium* (Kersters and De Ley, 1984a).

Since then, the relationships of nodulating, nitrogen-fixing species have been investigated by comparative analysis of 16S rDNA sequence data. Sequences of the type strains, obtained from international databases, have been subjected to various forms of algorithmic and parsimonious analysis in order to establish their phylogenetic relationships (Sawada et al., 1993; Willems and Collins 1993; de Lajudie et al., 1994, 1998b, 1998a; Nour et al., 1995; Rome et al., 1996; Young and Haukka 1996; Amarger et al., 1997; Tan et al., 1997; Lindström et al., 1998). Comparison of these analyses with others that have been published (Rome et al., 1996; Jarvis et al., 1997; de Lajudie et al., 1998a, b; van Berkum et al.,

TABLE 2. Characteristics of *Rhizobium*, *Agrobacterium*, *Allorhizobium*, and *Sinorhizobium* species[a, b]

Characteristics	*Rhizobium leguminosarum*	*Rhizobium etli*	*Rhizobium galegae*	*Rhizobium gallicum*	*Rhizobium giardinii*	*Rhizobium hainanense*	*Rhizobium huautlense*	*Rhizobium mongolense*	*Rhizobium tropici*	*Agrobacterium tumefaciens*	*Agrobacterium rhizogenes*	*Agrobacterium rubi*	*Agrobacterium vitis*	*Allorhizobium undicola*	*Sinorhizobium fredii*	*Sinorhizobium meticae*	*Sinorhizobium meliloti*	*Sinorhizobium saheli*	*Sinorhizobium terangae*	*Sinorhizobium xinjiangense*
Polar flagella			1–2			1													1	1
Peritrichous flagella or one subpolar flagellum	2–6		1–2					1	yes	1–4	1–4	1–4	1–4	yes	1–3		2–6	1	1	1–3
3-ketolactose produced	–		–	–	–	–			–	+	–	–	–	–	–		–	–	–	
Growth factors required:	+	–	+							–	+	+	+	–			+			
Biotin	d	–								–	+	+	+	–			d			
Pantothenate	+	–	+				–			–		+		–		–	–			
Thiamine	d	–	–				+			–				–		–	–			
pH range	4–9		5–9.5[c]	>4 to <8	4–8.5	5–10	5–9	4–10	4–10		5–9				5–10.5	5–10	4.5–9.5			5–10.5
Grows at 28°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grows at 35°C		+	+			+	+			+	–	–	–		+	+	+	+	+	+
Grows at 40°C	–	–	–	–	–	+	+		+		–	–	–		–	d	d	+	+	
Grows in 1% NaCl	–		d[c]	–	d	+	+	–	–	+			+		+	+	+			+
Grows in 2% NaCl	–		–	–	–	+	–	–	–	+	–	–	+		d	+	d			(+)
Growth in Luria–Bertani medium	–	–	–	–	–	+	–		+	+					–	+	+	–	–	–
Oncogenicity to few or many plant genera[d]	–	–	–	–	–	–	–		–	many[d]	many[d]	few[d]	few[d]	–	–	–	–	–	–	–
Symbiotic nodulating/nitrogen-fixing ability[d]	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+

[a]For symbols see standard definitions.
[b]Data are from Graham and Parker (1964), Jordan (1984a), Kersters and De Ley (1984a), Kerr (1992) and original descriptions of species.
[c]Unpublished data of E.T. Wang (personal communication) using the methods of Wang et al. (1998).
[d]Because oncogenicity and nitrogen-fixing symbioses are plasmid-mediated and the stability of resulting host specificity is also uncertain, these characteristics are not reliable bases for classification or identification of these species.

TABLE 3. Carbon source utilization tests which differentiate *Rhizobium*, *Agrobacterium*, *Allorhizobium*, and *Sinorhizobium* species[a,b]

Substrate[c,d]	*Rhizobium leguminosarum*	*Rhizobium galegae*	*Rhizobium tropici*	*Agrobacterium tumefaciens*	*Agrobacterium rhizogenes*	*Agrobacterium vitis*	*Allorhizobium undicola*	*Sinorhizobium fredii*	*Sinorhizobium meliloti*	*Sinorhizobium saheli*	*Sinorhizobium terangae*
Number of strains[e]	37	2	3	7	3	2	6	2	3	4	20
Acetate		+	d	+	–	+	d	–	d	+	+
N-acetylglucosamine		+	+	+	+	+	–	+	+	+	+
Aconitate		–	+	+	–	–	–	d	–	+	d
Adonitol		+	+	+	+	+	–	+	+	+	+
L-(alpha)-Alanine	+	+	+	+	+	+	+	–	d	+	d
beta-Alanine		–	–	–	d	–	d	–	+	d	–
DL-3-Amino butyrate		–	–	–	–	–	d	–	+	–	–
DL-4-Amino butyrate		–	d	d	–	+	d	+	+	d	+
DL-5-Aminovalerate		d	–	–	d	–	d	–	d	d	d
Amygdalin		–	+	–	+	d	–	–	–	–	d
D-Arabinose		+	+	+	+	d	–	d	+	+	+
L-Arabitol		–	+	+	+	–	–	+	+	–	–
Arbutin		+	+	–	+	+	+	+	+	+	+
L-Arginine	+	d	–	–	+	–	d	d	+	+	d
L-Aspartate		–	+	+	+	+	d	–	+	+	d
Butyrate		–	–	–	–	+	–	–	–	–	d
Citrate	–	d	d	–	+	+	–	–	–	–	–
L-Citrulline		–	d	–	+	–	–	d	–	+	–
L-Cysteine	+	–	–	–	d	–	–	–	–	–	–
Dulcitol	+	–	–	+	d	–	–	–	+	–	–
Erythritol	+	–	d	–	+	–	–	d	+	–	+
Ethanolamine		d	–	–	+	–	–	+	+	+	d
D-Fucose		+	d	+	+	+	d	d	d	–	–
Gluconate	+	+	+	+	+	–	–	–	–	d	d
L-Glutamate	+	–	+	+	d	d	d	+	+	+	+
Glutarate		–	d	d	–	+	–	–	–	–	–
DL-Glycerate		d	+	+	+	+	–	d	d	+	d

TABLE 3. (*Continued*)

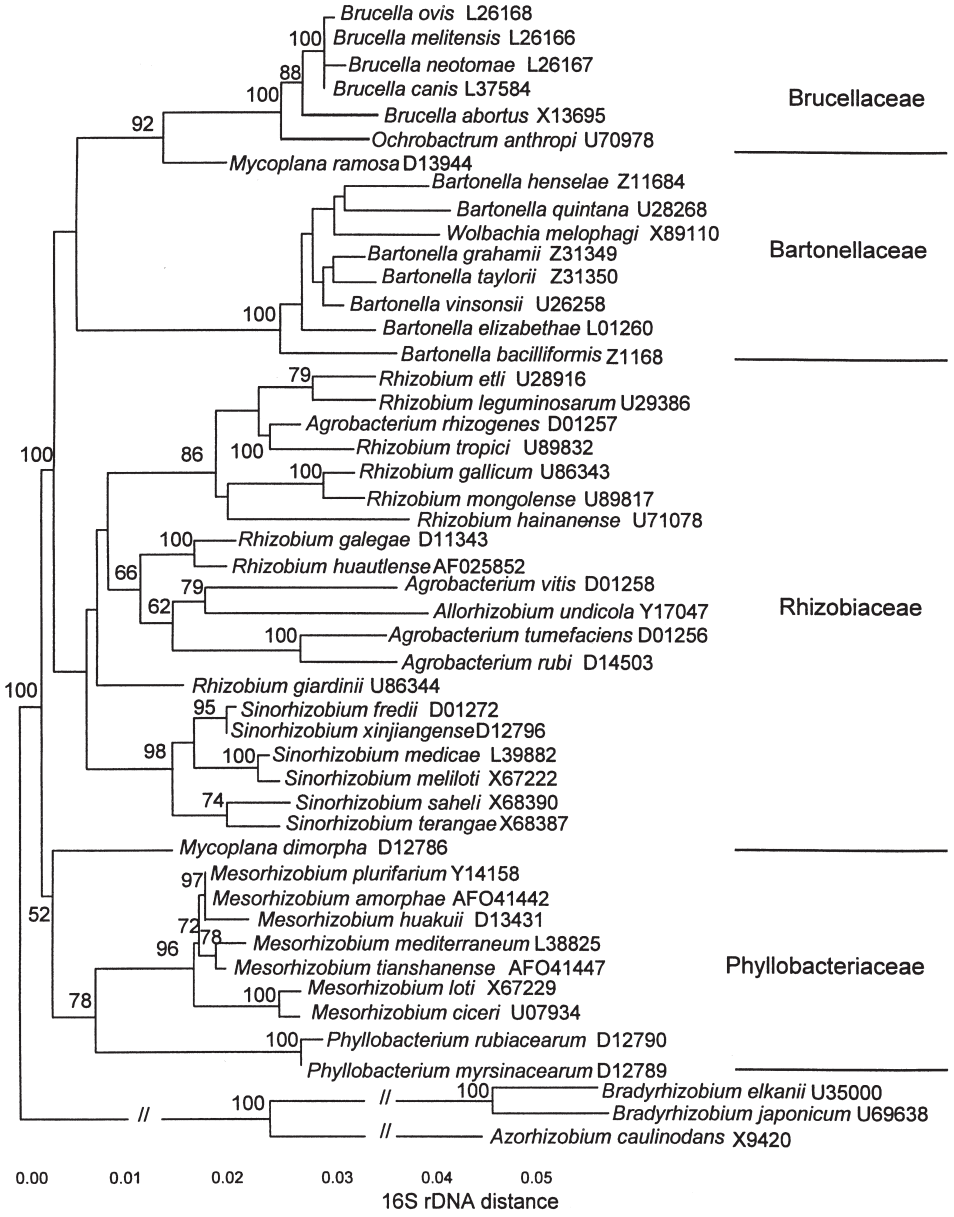
Substrate[c,d]	*Rhizobium leguminosarum*	*Rhizobium galegae*	*Rhizobium tropici*	*Agrobacterium tumefaciens*	*Agrobacterium rhizogenes*	*Agrobacterium vitis*	*Allorhizobium undicola*	*Sinorhizobium fredii*	*Sinorhizobium meliloti*	*Sinorhizobium saheli*	*Sinorhizobium terangae*
Glycolate		–	–	–	–	–	–	–	–	+	–
m-Hydroxybenzoate		+	–	–	–	–	–	–	–	–	–
p-Hydroxybenzoate		+	+	d	+	+	d	–	–	+	d
DL-3-Hydroxybutyrate		+	d	+	d	+	d	–	d	+	d
Isobutyrate		–	–	–	–	–	–	–	d	+	d
L-Isoleucine	+	–	–	–	d	–	–	–	d	d	–
2-Ketogluconate		+	–	+	+	d	d	–	+	d	+
5-Ketogluconate		–	–	+	+	–	–	–	–	–	–
2-Ketoglutarate		d	+	–	d	–	d	d	–	–	–
DL-Lactate	–	+	+	+	+	+	+	+	+	+	+
L-Leucine	+	d	d	d	d	–	–	d	d	d	+
L-Lysine	+	d	–	–	+	–	–	–	+	+	+
D-Lyxose		+	+	+	+	+	+	–	+	d	d
Malonate		–	d	–	–	–	–	–	–	–	–
D-Mandelate		–	+	–	–	–	–	–	d	–	–
L-Mandelate		–	+	–	–	–	–	–	–	–	–
D-Melibiose		+	+	+	+	+	–	+	+	+	+
D-Melezitose		–	–	+	–	–	–	d	+	d	d
Methyl-D-glycoside		d	+	–	+	–	–	–	+	–	d
Methyl-D-xyloside		–	+	+	+	–	–	+	+	–	–
L-Ornithine		d	d	–	+	–	+	+	+	+	+
L-Phenylalanine	+	+	–	–	–	–	–	d	–	d	–
Propionate		–	–	–	+	–	–	–	d	+	d
Pyruvate	–	+	+	+	+	+	(+)	d	+	+	(+)
D-Raffinose		+	+	+	+	+	–	+	+	+	+
Salicin		–	d	–	+	+	d	+	+	d	d
Sarcosine		–	d	–	+	–	–	–	–	–	–
L-Serine	+	+	d	–	+	d	+	–	d	+	d

TABLE 3. (*Continued*)

Substrate[c,d]	*Rhizobium leguminosarum*	*Rhizobium galegae*	*Rhizobium tropici*	*Agrobacterium tumefaciens*	*Agrobacterium rhizogenes*	*Agrobacterium vitis*	*Allorhizobium undicola*	*Sinorhizobium fredii*	*Sinorhizobium meliloti*	*Sinorhizobium saheli*	*Sinorhizobium terangae*
L-Sorbose		–	–	d	+	–	–	–	+	–	–
D-Tagatose		–	–	+	+	–	–	–	+	–	–
D-Tartrate		–	–	+	–	–	–	–	–	–	d
L-Tartrate		–	+	–	–	+	–	–	–	–	–
meso-Tartrate		–	–	+	–	–	–	–	–	–	d
L-Threonine	+	d	d	–	+	d	+	–	d	+	d
Trigonelline		–	+	–	d	+	d	d	+	d	–
L-Tyrosine	+	+	–	–	–	–	–	–	d	d	d
L-Valine	+	–	–	–	d	–	–	–	d	+	d
Xylitol		+	+	+	+	–	–	–	+	–	+
L-Xylose		–	+	d	+	d	–	–	d	d	d

[a]For symbols see standard definitions; (+), weak reaction.
[b]Carbon source utilization data is from de Lajudie et al. (1994) and de Lajudie et al. (1998a). Data for *R. leguminosarum* are from Amarger et al. (1997).
[c]Substrates which gave positive reactions by strains of all species: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, L-histidine, inositol, D-mannose, rhamnose, ribose, sorbitol, D-turanose, and D-xylose. Fumarate, glycerol, lactose, L-malate, maltose, mannitol, sucrose, succinate, and trehalose were substrates for which only one species expressed variable negative reactions.
[d]Substrates which gave negative reactions by strains of all species: adipate, D-("alpha")-alanine, DL-2-aminobutyrate, amylamine, azelate, benzoate, benzylamine, butylamine, caprate, *n*-caprate, caprylate, citraconate, diaminobutane, esculin, ethylamine, glycine, glycogen, heptanoate, histamine, *o*-hydroxybenzoate, inulin, isophthalate, isovalerate, itaconate, DL-kynurenine, levulinate, maleate, mesaconate, L-methionine, DL-norvaline, oxalate, pelargonate, phenylacetate, phthalate, pimelate, sebacate, spermine, starch, suberate, terephthalate, D-tryptamine, tryptophan, urea, and *n*-valerate. 2-Aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, L-cysteine, fumarate, methyl-D-mannoside, L-norleucine, and sucrose were substrates for which only one species expressed variable positive reactions.
[e]Data are included only where information is available for two or more strains.

FIGURE 3. Neighbor-joining tree expressing the relationships of genera within the *Rhizobiaceae* (*Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium* and related families) based on 16 S rDNA sequences. Bootstrap probabilities (as percentages) are determined from 1000 resamplings.



1998; Wang et al., 1998), and with Figure 3 of this chapter, shows how much results can vary depending on the selection of sequences and the form of analysis. Notwithstanding, all data clearly support the separation of *Bradyrhizobium* Jordan 1982 (now assigned to the family *Bradyrhizobiaceae*; Kuykendall, this volume) and *Azorhizobium* Dreyfus et al., 1988 (now assigned to the family *Hyphomicrobiaceae*; Kuykendall, this volume) as distantly related to all the other pathogenic and nodulating species. These latter species are allocated to the closely related families, *Rhizobiaceae* and *Phyllobacteriaceae*. Nitrogen-fixing and oncogenic (hypertrophying) species in *Agrobacterium*, *Rhizobium*, and *Sinorhizobium* are assigned to the *Rhizobiaceae* and species of *Mesorhizobium* (Jarvis et al., 1997; Chen and Kuykendall, this volume) are assigned to the *Phyllobacteriaceae* (Chen and Kuykendall, this volume).

The family *Rhizobiaceae* includes symbiotic and pathogenic species in the genera *Agrobacterium*, *Rhizobium*, and *Sinorhizobium*. Species allocated to these genera are found in

two or three clusters. One cluster comprises *Sinorhizobium*: *Sinorhizobium fredii* (the type species), *Sinorhizobium arboris*, *Sinorhizobium kostiense*, *Sinorhizobium medicae*, *Sinorhizobium meliloti*, *Sinorhizobium saheli*, *Sinorhizobium terangae*, and *Sinorhizobium xinjiangense*. *S. arboris* and *S. kostiense* were only recently described (Nick et al., 1999). The second cluster is more heterogeneous and may be considered to be represented by two subgroups. Subgroup 2a includes *R. leguminosarum* (the type species), *R. etli*, *R. gallicum*, *R. giardinii*, *R. hainanense*, *R. mongolense*, *R. tropici*, and *Agrobacterium rhizogenes*. Subgroup 2b includes *R. galegae*, *R. huautlense*, *Agrobacterium tumefaciens* (the type species), *Agrobacterium rubi*, *Agrobacterium vitis*, and *Allorhizobium undicola* (the type species). All these species have base differences amounting to less than 7% of the total 16S rDNA sequence. The extent of statistical support for individual branches and their relative positions depend on the form of phylogenetic analysis and the selection of sequences. Eardly et al. (1996), Martínez-Romero and Caballero-Mellado (1996), and Young and Haukka (1996) note anomalies in sequence analyses that are attributable to recombination events between species, a conclusion supported but qualified by Wernegreen and Riley (1999). As yet the significance and implications of recombination on the inference of phylogenetic relationships are unclear.

The family also contains, as outliers to the rhizobial species, strains named *Blastobacter* spp. and "*Liberibacter*" spp., which do not have symbiotic or pathogenic characteristics. Other strains of *Blastobacter* spp. are to be found in the families *Methylobacteriaceae* (four strains), *Bradyrhizobiaceae* (one strain), and *Sphingomonadaceae* (one strain). *Blastobacter aggregatus* ATCC 43293 and *Blastobacter capsulatus* ATCC 43294 in the *Rhizobiaceae* are therefore perhaps incorrectly named. The new genus "*Liberibacter*" represents strains of the fastidious organism that is the pathogen of citrus greening disease. This organism appears to be relatively distantly related to *Rhizobium*.

When first proposed, *Sinorhizobium* (Chen et al., 1988b) was based on only a small number of nutritional and biochemical tests, and its validity was questioned by Jarvis et al. (1992) on the basis of partial 16S rDNA sequence analysis and on the interpretation of numerical data. This genus has since been examined in greater detail and an emended circumscription of the genus has been produced (de Lajudie et al., 1994). However, this circumscription does not delineate a taxon distinct from *Rhizobium*, and the polyphasic data reported (PAGE of total proteins and carbon source utilization tests) do not support a coherent taxon. Moreover, the protein data show *Sinorhizobium fredii* (the type strain) as an outlier to the other species, and carbon source utilization data show *Sinorhizobium* species intermingled with *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium*. Support for this genus, distinct from *Rhizobium*, is based on comparative 16S rDNA sequence data alone (de Lajudie et al., 1994).

Allorhizobium, is a monospecific genus established because—as indicated by its name—comparative analysis of 16S rDNA sequence data for *Allorhizobium undicola* indicated that this organism belonged to an outlying branch (de Lajudie et al., 1998b). The closest neighboring species in their analysis was *Agrobacterium vitis*. The species *Allorhizobium undicola* is well defined based on DNA–DNA reassociation, PAGE of total proteins, and carbon source utilization tests. As with *Sinorhizobium*, the circumscription of the genus does not delineate a taxon distinct from *Rhizobium*, and the polyphasic data do not support a close relationship between *Allorhizobium undicola* and *Agrobacterium vitis* and other agrobacteria. In proposing a new genus, rather than either nominating the species as a nitrogen-fixing member of *Agrobacterium ex tempore* or allocating it to *Rhizobium ex tempore*, the authors are committed to a nomenclature in which either *Agrobacterium vitis* is renamed *Allorhizobium vitis* or is recognized in its own monospecific genus. While monospecific genera can sensibly be named when there is clear justification based on a unique circumscription of the taxon, the practice of naming taxa when they are merely outlying members of larger clades must be questioned. In such cases, where the description of the genus is indistinguishable from adjacent genera and where the species description forms the basis of the generic description, there is no basis for forestalling an on-going process of naming monospecific genera across the clade. This approach to classification implies the creation of many genera containing small numbers of species with circumscriptions indistinguishable from *Rhizobium*.

In regard to "rhizobia," legume plants are represented by more than 14,000 species (Jordan 1984a; Lindström et al., 1998), of which fewer than 1% have been investigated to establish the identity of their associated nitrogen-fixing bacterial species. Considering the relatively close relationships of taxa within the *Rhizobiaceae*, it is highly probable that new taxa will be characterized that will be intermediate between the present named species and genera (*Agrobacterium*, *Rhizobium*, and *Sinorhizobium*). Intermediate taxa can be expected to obscure the apparent deep branches between currently named genera (Martínez-Romero and Caballero-Mellado, 1996).

Murray et al. (1990) have made clear the expectation that at the generic level, taxa should be supported with phenotypic descriptions. Proposed minimal standards for *Agrobacterium*

and *Rhizobium* require that generic as well as species names should be based on both phenotypic and phylogenetic data (Graham et al., 1991). Phylogenetic divisions can only be understood as generating distinct genera where these have evolved into discrete phenotypic groups. Genera differentiated solely on sequence data have the same nomenclatural status as taxa erected as *Candidatus*, as described by Murray and Stackebrandt (1995). Furthermore, 16S rDNA sequence data cannot be accepted uncritically where there is evidence of recombination within sequences (Eardly et al., 1996; Martínez-Romero and Caballero-Mellado, 1996; Young and Haukka, 1996).

With the exception of the 16S rDNA-based discrimination of *Sinorhizobium* as a distinct clade, the generic circumscriptions of *Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium* contain no demonstrated characteristics by which these genera can be supported as distinct taxa. Multiple character analysis towards a phenetic (Goodfellow and O'Donnell, 1993; Goodfellow et al., 1997) or polyphasic (Vandamme et al., 1996b) classification aims to produce coherent taxa with relevant circumscriptions. Electrophoretic protein patterns, and numerical analysis of nutritional and biochemical data (de Lajudie et al., 1994) gave no support for segregation of species into the separate genera *Agrobacterium*, *Rhizobium*, and *Sinorhizobium*. Terefework et al. (1998) and de Lajudie et al. (1998a) provide data showing that various pathogenic and nodulating, symbiotic bacteria belonging to the genera *Agrobacterium* and *Rhizobium* are interspersed on subbranches of 16S or 23S phylogenetic trees. Analysis of fatty acid profiles showed that these three genera were closely related (Jarvis et al., 1996), but that species of *Mesorhizobium* formed a distinct group. More recently, Tighe et al. (2000) showed that the concentration of $C_{16:0\ 3OH}$ fatty acid was generally somewhat lower in *Sinorhizobium* than *Rhizobium*. 16S rDNA sequence data clearly show that *Agrobacterium* spp. (Willems and Collins, 1993) are closely related to *Rhizobium* spp. such as *Rhizobium galegae* (Lindström, 1989), and the recently described *Rhizobium huautlense* (Wang et al., 1998). *Agrobacterium rhizogenes* is always found among authentic *Rhizobium* spp. Many past studies have suggested the need for amalgamation of *Agrobacterium* and *Rhizobium* (Graham 1964, 1976; Heberlein et al., 1967; De Ley, 1968; White, 1972; Kerr, 1992; Sawada et al., 1993). It has long been clear that pathogenic (*Agrobacterium*) and nitrogen-fixing (*Rhizobium*) species are interspersed, and there seems to be a paucity of justification for the on-going separation of these genera. *Agrobacterium* is a polyphyletic genus that is an artificial amalgamation of plant-pathogenic species (Young et al., 2001).

The four named genera—*Agrobacterium*, *Allorhizobium*, *Rhizobium*, and *Sinorhizobium*—are closely related in genomic and phenotypic terms. It has recently been proposed by the authors of this treatment that *Agrobacterium*, *Allorhizobium*, and *Rhizobium* be amalgamated into a single genus, *Rhizobium*, based on their close genomic and phenotypic similarity (Young et al., 2001). We maintain that *Allorhizobium* is an artificial genus and in phylogenetic terms is part of the *Agrobacterium*/*Rhizobium* cluster. Indeed it seems clear that its creation was only justified based on the anomalous state of *Agrobacterium* nomenclature at the time it was proposed by de Lajudie et al., (1998a). The branches between *Agrobacterium* and *Rhizobium* on the one hand and *Sinorhizobium* on the other suggest an evolutionary divergence that could be interpreted as preliminary to the formation of new genera. At present these two clades are probably incipient genera and merit no more than recognition as subgenera.[2]

Differentiation of the species of the genus *Rhizobium*

Morphological, biochemical, and nutritional data are given in Tables 2 and 3. Nutritional data have been reported for only about half of *Rhizobium* species (Table 3). Data are included here only if more than one strain for each species has been reported. Some recently named species have been established largely using comparative 16S rDNA sequence analyses. Phenotypic descriptions of these species, as well as a comprehensive comparative investigation of the whole genus, are needed.

The phenotypic descriptions of the symbiotic nodule-forming *Rhizobium* species are based on D.C. Jordan's treatment in the first edition of *Bergey's Manual of Systematic Bacteriology* in 1984 and on original descriptions of new species. The phenotypic description of *Agrobacterium* is based on studies by De Ley et al. (1966), Lippincott and Lippincott (1969), Keane et al. (1970), White (1972), Kersters et al. (1973), Panagopoulos and Psallidas (1973), Kerr and Panagopoulos (1977), Süle (1978), Panagopoulos et al. (1978), Holmes and Roberts (1981), Sawada and Ieki (1992b), de Lajudie et al. (1994), and Amarger et al. (1997).

List of species of the genus *(Rhizobium)*

Rhizobium leguminosarum
(Frank 1879) Frank 1889, 338[AL] (*Schinzia leguminosarum* Frank 1879, 397.)

le.gu.mi.no.sa'rum. M.L. fem. n. *Leguminosae* old family name of the legumes; M.L. gen. pl. n. *leguminosarum* of legumes.

The characteristics are as given for the genus and listed in Tables 2 and 3. The cells have either 1 or 2 polar flagella or 2–6 peritrichous flagella. Growth does not occur at 39–40°C. The pH range for growth is 4.5–9.0. Growth does not occur in YMA containing 2% NaCl. Pantothenate and, for some strains, thiamine are required as growth factors. Well characterized genetically. Three biovars of *R. leguminosarum* have been defined, biovar trifolii (previously classified as *Rhizobium trifolii* Dangeard 1926[AL]), biovar phaseoli, and biovar viceae, based on nodulating specificity. Selected strains of biovar phaseoli have been elevated to the status of species as *R. etli*, *R. gallicum*, *R. giardinii*, *R. mongolense*, and *R. tropici*. Residual strains representing *R. leguminosarum* need to be re-examined and the description of the species needs to be emended.

R. leguminosarum nodulates with some, but not necessarily all, *Lathyrus* spp., *Lens* spp., temperate species of *Phaseolus* (*P. vulgaris*, *P. angustifolius*, *P. multiflorus*), *Pisum* spp., *Trifolium* spp., and *Vicia* spp.

The mol% G+C of the DNA is: 59–63 (T_m).

Type strain: ATCC 10004, DSM 30132, NCIB 11478, USDA 2370.

GenBank accession number (*16S rRNA*): U29386.

Rhizobium etli
Segovia, Young, and Martínez-Romero 1993,376[VP]

et' li. L. n. *etl* bean; N.L. gen. n. *etli* of bean.

The characteristics are as given for the genus and listed in Table 2. Fast growing: colonies are 2–4 mm in diameter after 2–4 d on peptone–yeast extract agar. No growth occurs on Luria broth medium or on peptone yeast-extract medium lacking calcium. Growth occurs on a minimal medium containing malate as a carbon source. Maximum temperature for growth is 37°C. Selected strains of *R. leguminosarum* biovar phaseoli were differentiated as this new species on the basis of differences in protein profiles, antibiotic resistance profiles, serological types, DNA–DNA reassociation data, plasmid profiles, exopolysaccharide structures, and multilocus enzyme electrophoresis.

The species contains two named biovars: *Rhizobium etli* biovar phaseoli and biovar mimosae (Wang et al., 1999a). The species nodulates and fixes nitrogen in association with *Phaseolus vulgaris* and some other legumes, such as *Mimosa affinis*. Nonsymbiotic strains are included in the species.

The mol% G+C of the DNA is: 59-63 (T_m).

Type strain: CFN 42, ATCC 51251, ICMP 13642, USDA 9032.

GenBank accession number (*16S rRNA*): U28916.

Rhizobium galegae
Lindström 1989, 365[VP]

ga.le' gae. M.L. fem. gen. n. *galegae* of *Galega*, a genus of leguminous plants.

The characteristics are as given for the genus and listed in Tables 2 and 3. Motile by 1–2 polar or subpolar flagella. Relatively slow growing. Colonies on YMA are more than 1.0 mm in diameter after 7 d at 28°C. Growth does not occur on YMA containing 2% NaCl. Maximum temperature for growth is 33–37°C. Most strains form a serum zone and give an alkaline reaction in litmus milk. Hydrolyzes urea but does not precipitate calcium glycerophosphate or reduce nitrate. Requires pantothenate as a vitamin supplement or growth factor, but not thiamine. Utilizes relatively few organic substrates as sole sources of carbon (Table 3). A preliminary report gave information on this species (Lindström and Lehtomäki, 1988). Nodulates *Galega orientalis* and *Galega officinalis* and is reported to be specific to these species.

The mol% G+C of the DNA is: 63 (T_m).

Type strain: HAMBI 540, ATCC 43677, DSM 11542, ICMP 13643, LMG 6214.

GenBank accession number (*16S rRNA*): D11343, X67226.

Rhizobium gallicum
Amarger, Macheret, and Laguerre 1997, 1005[VP]

gal' li.cum. L. adj. *gallicum* pertaining to Gallia; the country of origin, France.

The characteristics are as given for the genus and listed in Table 2. Relatively fast growing. Colonies are 2–4 mm in diameter within 2–3 d at 28°C. Growth does not occur on YMA containing 1% NaCl. Resistant to nalidixic acid. Separated from other *Rhizobium* species by cluster analysis of phenotypic data. The status of *R. gallicum* as an authentic species is supported by amplified 16S rDNA restriction analysis, comparative 16S rDNA sequence analysis, DNA–DNA reassociation, and nutritional data.

Two biovars, *R. gallicum* biovar gallicum and *R. gallicum* biovar phaseoli, are established based on nodulating specificity. *R. gallicum* biovar gallicum nodulates and fixes nitrogen in association with *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Onobrychis viciifolia*, and *Phaseolus* spp. whereas *R. gallicum* biovar phaseoli nodulates *Phaseolus* spp. only.

The mol% G+C of the DNA is: not available.

Type strain: R602sp, MSDJ1109.

GenBank accession number (*16S rRNA*): AF008130, U86343.

Rhizobium giardinii
Amarger, Macheret, and Laguerre 1997, 1005[VP]

giar.di'ni.i. N.L. gen. n. *giardinii* of Giardini, a Brazilian microbiologist who isolated the organism.

The characteristics are as given for the genus and listed in Table 2. Relatively fast growing. Colonies are 2–4 mm in diameter within 2–3 d at 28°C. Growth does not occur on YMA containing 2% NaCl. Resistant to nalidixic acid. The status of *R. giardinii* as an authentic species is supported by amplified 16S rDNA restriction analysis, DNA–DNA hybridization, and comparative 16S rDNA sequence analysis.

Two biovars, *R. giardinii* biovar giardinii and *R. giardinii* biovar phaseoli, are established based on nodulating specificity. *R. giardinii* biovar giardinii nodulates *Phaseolus* spp., *Leucaena leucocephala*, and *Macroptilium atropurpureum*, but does not fix nitrogen with *Phaseolus vulgaris*. *R. giardinii* biovar phaseoli nodulates *Phaseolus* spp. and is weakly efficient in fixing nitrogen in association with that host.

The mol% G+C of the DNA is: not available.

Type strain: H152, MSDJ0144.

GenBank accession number (*16S rRNA*): U86344.

Rhizobium hainanense
Chen, Tan, Gao, Li, and Wang 1997b, 872[VP]

hai.na.nen'se. M.L. neut. adj. *hainanense* pertaining to Hainan Province in China.

The characteristics are as given for the genus and listed in Table 2. Motile by a single polar flagellum. Relatively fast growing, with a generation time of 2–4 h. Colonies are 2–4 mm in diameter after 3 d growth on yeast extract mannitol agar. Temperature optimal for growth, 25–30°C. Strains can grow at 40°C. Optimal pH for growth, 6–8; pH range, 5–10. Grows on YMA containing 2% NaCl.

The status of *R. hainanense* as an authentic species is supported by cluster analysis of phenotypic features, DNA–DNA reassociation data, and comparative 16S rDNA sequence analysis.

Nodulates *Acacia sinicus*, *Arachis hypogaea*, *Centrosema pubescens*, *Desmodium gyroides*, *D. sinuatum*, *D. triquetrum*, *D. heterophyllum*, *Macroptilium lathyroides*, *Stylosanthes guianensis*, *Tephrosia candida*, *Uraria crinita*, and *Zornia diphylla*.

The mol% G+C of the DNA is: 59–63 (T_m).

Type strain: 166, CCBAU 57015, DSM 11917, ICMP 13690.

GenBank accession number (*16S rRNA*): U71078.

Rhizobium huautlense
Wang, van Berkum, Beyene, Sui, Dorado, Chen and Martínez-Romero 1998, 696[VP]

hu.aut.len'se. N.L. adj. *huautlense* of Huautla, the region in Mexico where the organisms were isolated.

The characteristics are as given for the genus and listed in Table 2. Relatively fast growing, with a generation time of 2.0–2.2 h. Colonies are 2–4 mm in diameter within 2–3 d at 28°C. Grows at 40°C. Growth does not occur on YMA containing 2% NaCl. Requires thiamine as a growth factor. Comparative sequence analysis of 16S rDNA indicates that this species is closely related to *R. galegae*. These two species are differentiated based on multilocus enzyme electrophoresis, DNA–DNA reassociation, size of compatible Sym plasmids, and a small number of other features. Nodulates *Sesbania herbacea*, *S. rostrata*, and *Leucaena leucocephala*.

The mol% G+C of the DNA is: 57–59 (T_m).

Type strain: S02, ICMP 13551, USDA 4900.

GenBank accession number (*16S rRNA*): AF025852.

Rhizobium lupini
(Schroeter 1886) Eckhardt, Baldwin and Fred 1931, 273[AL] (*Phytomyxa lupini* Schroeter 1886, 135.)

lu.pi'ni. M.L. masc. n. *Lupinius* generic name of lupine; M.L. gen. n. *lupini* of *Lupinus*.

A limiteddescription is given in Jordan and Allen (1974). Jordan (1984a) recognized the affinities of this species with *Bradyrhizobium* species but felt that evidence was lacking to propose the transfer of *R. lupini* to the genus. Since then comparative sequence analyses of 16S rDNA from nodulating strains of *Lupinus* support transfer of the species; however, the 16S rDNA sequence of the type strain, which has unfortunately been contaminated with *B. japonicum* but still contains another distinct species (van Berkum et al., 1998), appears not to have been analyzed. The status of this species clearly needs work.

Nodulates *Lupinus* spp. and *Ornithopus* spp. Limited nodulation of *Glycine* spp. and the cowpea miscellany.

The mol% G+C of the DNA is: not available.

Type strain: ATCC 10319, DSM 30140.

Rhizobium mongolense
van Berkum, Beyene, Bao, Campbell and Eardly 1998, 21[VP]

mon.go.len'se. L. neut. adj. *mongolense* pertaining to Inner Mongolia, the region where the bacteria were isolated.

The characteristics are as given for the genus and listed in Table 2. Motile by a single polar or subpolar flagellum. Relatively fast growing: colonies 1–3 mm in diameter within 3–5 days on arabinose-gluconate agar. No growth occurs on YMA containing 1% NaCl or at pH values below 4.0. Resistant to bacitracin, cefuperazone, and penicillin G. *R. mongolense* shares 99.2% similarity in its 16S rDNA sequence with *R. gallicum* (van Berkum et al., 1998), and it may therefore be a junior synonym of *R. gallicum*. Isolated from *Medicago ruthenica*. Nodulates *Medicago ruthenica* and *Phaseolus vulgaris*.

The mol% G+C of the DNA is: not available.

Type strain: USDA 1844 (ICMP 13688).

GenBank accession number (*16S rRNA*): U89817

Rhizobium tropici Martínez-Romero, Segovia, Mercante, Franco, Graham and Pardo 1991, 424^{VP}

tro'pi.ci. M.L. gen. n. *tropici* of the tropic (of Cancer).

The characteristics are as given for the genus and listed in Tables 2 and 3. Motile by peritrichous flagella. Relatively fast growing, with a doubling time of 1.6–2.0 h. Colonies are 2–4 mm in diameter within 2–4 days at 30°C on mannitol–yeast extract agar and peptone yeast-extract agar. Can grow at 40°C. Growth occurs at pH 4.5–7. Does not produce 3-ketolactose. Utilizes a range of organic substrates as sole sources of carbon (Table 3).

Previously classified as Type II strains of *Rhizobium leguminosarum* biovar phaseoli. This species comprises Type A and Type B strains, which may represent two distinct species. Distinguished from *Rhizobium leguminosarum* by host range, *nif* gene organization, high temperature tolerance, and extreme acid tolerance, and are said to be more symbiotically stable. Distinguished from other *Rhizobium* species by DNA–DNA reassociation, multilocus enzyme electrophoresis profiles, in biochemical tests, and 16S rDNA sequence comparison.

Forms nodules on *Phaseolus vulgaris*, *Leucaena* spp., and with other legume species. Type A strain CFN299 also nodulates *Amorpha fruticosa*.

The mol% G+C of the DNA is: 60–62 (T_m).

Type strain: ATCC 49672, ICMP 13646, IFO 15427, LMG 9503, USDA 9030.

GenBank accession number (*16S rRNA*): U89832, X77125.

End notes

1. Yeast extract-mannitol agar (YMA) contains (g/l of distilled water): D-mannitol, 10.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCO_3$, 4.0; yeast extract (Difco), 0.4; agar, 15.0; pH 6.8–7.0. Sterilize at 121°C for 15 or 30 min depending on the volume. The $CaCO_3$ is omitted for the preparation of pour plates or for liquid medium.
2. Since the completion of this manuscript, a proposal to include species of *Agrobacterium*, *A. radiobacter*, *A. rhizogenes*, *A rubi*, *A. vitis*, and *Allorhizobium undicola* in *Rhizobium* with an emended description of the genus, has been made (Young et al., 2001), as a more natural polyphasic, interpretation of the the taxonomy of the family *Rhizobiaceae*. The status of the genus *Sinorhizobium* is considered to need further evaluation.

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