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Review article
Coevolution of Rhizobia with Legumes: Facts and Hypotheses

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Abstract

The ability to form N_2-fixing symbioses has a monophyletic (paraphyletic) origin in legumes and a polyphyletic origin in rhizobia. Analysis of taxonomic data suggests that in legumes nodulation is a more ancient property than symbiotrophic nitrogen nutrition while in rhizobia the ability to nodulate their hosts is of a more recent origin than the ability to fix N_2. The ancestors of both partners are supposed to have possessed the preadaptations which ensured a subsequent evolution of the symbiotic system. The plant preadaptations might have been: spontaneous formation of the nodule-like structures and an ability to permit a persistence of potential symbionts in them. The bacterial preadaptations might have been: the ability to fix N_2 and to resist (escape) the plant defense reactions. In rhizobia the nodulation ability is supposed to evolve mainly via the individual selection, while the ability for symbiotic N_2 fixation – via the kin selection of the clones. Evolution of *nod* genes in rhizobia is supposed to elicit formation of the mechanisms for restriction (regulation) of nodulation in hosts. Although the legume-rhizobia coevolution for nodulation might have the marked similarities with the gene-for-gene coevolution, the direct evolutionary relationships between legume-rhizobia symbioses and the phytopathogenic systems are not probable. The plant-bacteria coevolution is suggested to be based on: (i) intensive intergenomic recombination and horizontal transfer of genes in rhizobia populations; (ii) increasing the genetic heterogeneity of these populations via interaction with the hosts.

Keywords: Nodulation, nitrogen fixation, legumes, rhizobia, coevolution, preadaptation, kin selection, gene-for-gene interactions

1. Introduction

The symbiosis between nodule bacteria (rhizobia) and legumes is the best studied plant-microbe N_2-fixing system. It attracts much attention because rhizobia (genera *Azorhizobium, Bradyrhizobium, Rhizobium, Sinorhizobium*) are facultative symbionts which may be analyzed by the genetic and molecular methods used for free-living organisms. The multistage process of nodule ontogeny is a fine model for analyzing the inter-organism interactions, plant molecular biology and developmental genetics. Besides, the legume-rhizobia symbiosis is of a great agricultural importance as a cheap and ecologically safe source of nitrogenous compounds.

The symbiosis establishment involves two basic processes – nodulation and nitrogen fixation. The former process includes: induction of the nodule primordia in root cortex, penetration of bacteria into the roots, differentiation of the nodule tissues and (in the majority of studied legumes) endocytosis of bacteria into the plant cells resulting in the symbiosome formation (Brewin, 1991; Pawlowski and Bisseling, 1996). The latter process includes: synthesis of nitrogenase and other bacterial enzymes participating in N_2 fixation, establishment of an integrated metabolic system in nodules where the plant-derived photosynthates are "exchanged" for the bacteria-derived NH_4^+ (Werner, 1992; Streeter, 1995). The partners' interactions are highly specific: each *Rhizobium* species can form an "effective" symbiosis (in which N_2 fixation is intensive enough to support the normal plant growth) with a defined group of legume hosts. e.g., *R. leguminosarum* bv. *viceae* is a symbiont of pea and vetch, *S. meliloti* of alfalfa and sweet clover, *B. japonicum* of soybean and siratro.

A huge volume of data accumulated on genetics, biochemistry, cytology and ecology of the legume-rhizobia symbiosis constitutes a basis for reconstructing its origin and evolution (Norris, 1956; Young and Johnston, 1989; Sprent and Raven, 1992; Sprent, 1994). Nowadays this symbiosis is the best developed model for analyzing the coevolutionary processes which play a crucial role in the life history (Van Valen, 1983). Here the hypotheses and facts are reviewed which throw light on the major stages and mechanisms of the legume-rhizobia coevolution.

2. Origin and Macroevolution of the Legume-Rhizobia Symbioses

Taxonomic aspects

The "rhizobia" are taxonomically diverse group of α-*Proteobacteria* which diversified from their hypothetical "common ancestor" long before the legume

hosts originated. The *(Sino)Rhizobium* species are related to agrobacteria (*Azo-*, *Bradyrhizobium*) and to different free-living diazotrophs (*Beijerinkia*, *Nitrobacter*, *Rhodopseudomonas*) (Martinez, 1994; Young and Haukka, 1996). In contrast, the legumes comprise a monophyletic (or a paraphyletic) group of dicotyledonous plants, the great majority of which are nodulated by rhizobia (Allen and Allen, 1981; Yakovlev, 1991). Only one non-legume genus, *Parasponia* (Ulmaceae) displays this property under natural conditions (Becking, 1992). Therefore, the ability for symbiosis has a monophyletic (paraphyletic) origin in legumes and a polyphyletic origin in rhizobia.

Two approaches are now available for reconstructing origin and macroevolution of the symbiotic system: a) to analyze the relationships between taxonomy and symbiotic properties in legumes; b) to compare different rhizobia for organization of "symbiotic" (*sym*) genes.

Symbiotic properties in different legume taxa. The ability to nodulate was revealed in 84% and 92% of the investigated Leguminosae genera and species, respectively (Allen and Allen, 1981). In the evolutionary "advanced" subfamilies (Mimosoideae, Papilionoideae) and tribes the nodulation is often (more than 90% of species nodulate) while in the "primitive" ones it is comparatively rare (e.g., in sbf. Caesalpinioideae only 30% of species nodulate). These data suggest that the legume progenitors had some preadaptation(s) which elicited a parallel evolution of the symbioses in different branches of Leguminosae (Young and Johnston, 1989; Doyle and Doyle, 1997; Sprent, 1997).

It is important to note that the infection and nodule morphology vary greatly in legumes (Sprent and Raven, 1992; Sprent et al., 1993; Pawlowski and Bisseling, 1996). First, the plants may be infected either through root hairs (alfalfa, pea, soybean) or through cracks in root epidermis (peanut, stylosanthes). Second, the root nodules are developed from the meristematic zones initiated *de novo* in either inner or outer cortex while the stem nodules in some tropical legumes (*Sesbania*, *Aeshynomene*) are suggested to develop from dormant primordia (Tsien et al., 1983). Third, different morphological types of nodules were identified with respect to location of the vascular system (stem-like or root-like nodules) or to persistence of the meristematic zone (determinate or indeterminate nodules). Fourth, the intracellular symbiosis is not characteristic for all legumes. In the nodules of some caesalpinioids and of the non-legume *Parasponia*, rhizobia do not penetrate the plant cells and fix N_2 in the infection threads sometimes called "fixation threads" (Sprent et al., 1993). Analysis of the taxonomic and morphological data suggests that the symbiosis may have originated no less than three times in the legume evolution (Sprent, 1997; Doyle and Doyle, 1997).
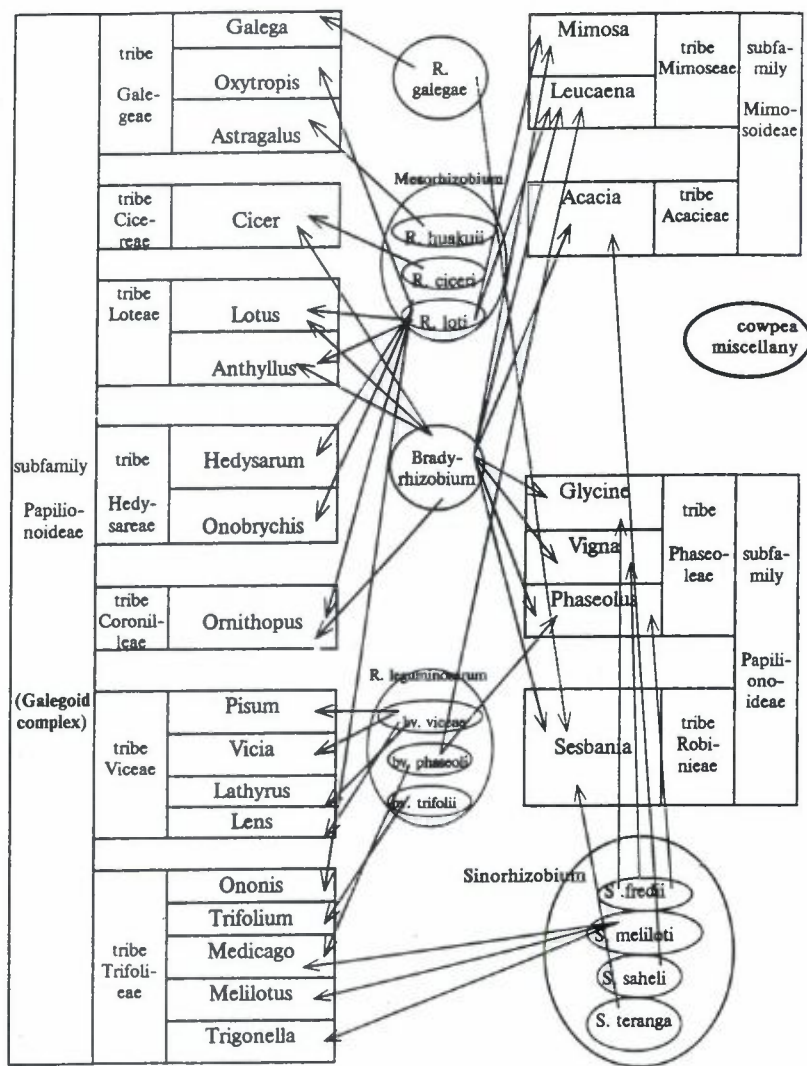
Figure 1. Cross-inoculation between legumes from the "galegoid complex" and "cowpea miscellany". The plant taxonomy is taken from: Yakovlev, 1991. Arrows show a possibility of formation of Fix$^+$ nodules (dotted arrows mean that the taxonomic position of the strains nodulating a given legume have been determined in preliminary tests). Data on host specificity are taken from: *R. galegae* – Lindstrom et al., 1983; Rana and Krishnan, 1995; *R. loti-R huakuii-R. ciceri* complex (*Mesorhizobium*) – Jensen, 1967; Jensen and Hansen, 1968; Chen et al., 1991; Novikova et al., 1993; Lindstrom et al., 1995b; *R. leguminosarum* bv. *phaseoli, R. etli, R. tropici* – Martinez-Romero et al., 1991; Hernandez-Lucas et al., 1995; *S. fredii* – Chen et al., 1988; Sadowsky et al., 1988; *S. saheli, S. teranga* – De Lajudie et al., 1994; for the other species the reviewed data (Vincent, 1967; Jordan, 1984; Provorov, 1994) were used.

The specificity of nodule formation (studied using the rhizobia strains isolated from different plant species) correlates markedly with the legume taxonomy, while the specificity of effective symbiosis formation does not correlate (Lieberman et al., 1985; Provorov, 1994). Therefore, nodulation seems to be a more ancient property of legumes than the symbiotrophic nitrogen nutrition.

An extensive cross-inoculation between the evolutionary advanced legumes of temperate zones (representing the galegoid complex, sbf. Papilionoideae) and the tropical legumes of the "cowpea miscellany" (representing diverse taxa of the Leguminosae) have been reported (Fig. 1). There is no correlation between phylogenies of legumes and rhizobia (Young and Johnston, 1989; Fig. 1). These data suggest a singularity of the mechanisms of rhizobia-legume coevolution because a striking concordance between phylogenies of partners have been revealed in many symbioses, both being mutualistic (Hinkle et al., 1994; Du et al., 1994; Baumann et al., 1997) and antagonistic (Hafner and Nadler, 1988).

Symbiotic (sym) genes in different taxa of rhizobia. These genes may be divided into several categories: (i) nodulation genes (*nod, nol, noe*) controlling the root penetration and infection thread formation (Rhijn and Vanderleyden, 1995; Pueppke, 1996); (ii) nitrogen fixation genes which encode: the synthesis of nitrogenase (e.g., *nifH, nifD, nifK, nifA, fixLJ, fixK*); the energy support of N_2 fixation, e.g., the components of electron (*cycY, fixX, fixNOQP*) and dicarboxylate (*dctA, dctB, dctD*) transport systems (Jording et al., 1994; Vargas et al., 1994; Fisher, 1994); (iii) genes for synthesis of exo- or lipopolysaccharides (*exo, exp, lps*) and cyclic glucans (*ndv*) which are essential for cellular interactions of partners (Leigh and Walker, 1994; Breedveld and Miller, 1994). In the majority of *(Sino)Rhizobium* species nodulation and nitrogen fixation genes are located on *sym* plasmids (pSyms), while a chromosomal location of these genes is typical for *Azorhizobium, Bradyrhizobium, R. loti* and some strains of *S. fredii* (Martinez et al., 1990; Santos et al., 1996).

The nodulation gene system comprises a principal difference of rhizobia from other plant-associated bacteria. The best studied function of these genes is synthesis of Nod factors which have a common lipo-chito-oligosaccharide (LCO) core structure but vary in number of monosaccharide residues, length of the unsaturated fatty acid chain and in "decorations" with acyl, sulfate, carbamoil and other groups (Rhijn and Vanderleyden, 1995; Schultze and Kondorosi, 1996; Pueppke, 1996). The Nod factors excreted by rhizobia elicit the early symbiotic reactions in plants, e.g., deformations of the root hairs, synthesis of the early nodulins, induction of the nodule meristems (Spaink, 1995; Long, 1996; Heidstra and Bisseling, 1996).

Only few (*nodA, nodB, nodC, nodD* and possibly *nodI, nodJ, nodS, nodU*) out of more than 50 discovered nodulation genes are "common" (structurally and functionally homologous) for all rhizobia species. *nodABC* are responsible for synthesis of a core part of Nod factors while *nodD* encodes a LysR-like protein which regulates transcription of other nodulation genes in response to the plant-excreted flavonoids. However, many nodulation genes are specific for particular rhizobia species (strains) providing infection of their hosts due to the defined modifications of Nod factors. e.g., *nodX* controls acetylation of the *R. leguminosarum* bv. *viceae* Nod factors enabling nodulation of "afghan" peas (Downie et al., 1994), *nodPQ* and *nodH* encode sulfation of the *S. meliloti* Nod factors permitting the alfalfa nodulation (Roche et al., 1991). Moreover, no correlation has been found between the sequence diversities of *nodABCD* genes and rhizobia taxonomy (Vazquez et al., 1991; Dobert et al., 1994; Ueda et al., 1995). These data suggest that the nodulation ability in rhizobia originated recently, during their coevolution with the legume hosts.

Quite different suggestions should be made from a comparative analysis of *nif* (*fix*) genes responsible for N_2 fixation. The majority of them are common to diverse rhizobia species (Fisher, 1994). Only few genes controlling a host specificity of N_2 fixation were reported (Crank et al., 1993; Chun et al., 1994). A pronounced correlation has been found between the rhizobia taxonomy (16S rDNA diversity) and *nif* gene diversity (Kaluza et al., 1985; Badenoch-Jones et al., 1989; Young, 1992; Dobert et al., 1994). These data suggest that N_2-fixing capacity is an ancient property of rhizobia which predisposed their progenitors for coevolution with the legume hosts.

From mutualism or from antagonism?

A possible origin of legume-rhizobia symbioses from antagonistic plant-bacteria interactions is broadly discussed. Several lines of evidence support this concept.

1) The *(Sino)Rhizobium* species are closely related to agrobacteria in phenotypic properties and genetic organization (Jordan, 1984; Martinez, 1994). The intermediate genotypes combining the properties of these two microbial groups may be found in soils, e.g., a *R. leguminosarum* bv. *trifolii* strain which forms N_2-fixing nodules on clovers and slow-growing tumors on tomatoes (Skotnicki and Rolfe, 1978). There are extended regions of homology between Ti-plasmids controlling oncogenicity of agrobacteria and Sym plasmids. However, these regions do not cover the genes essential for the plant interactions (Prakash and Schilperoort, 1982). Some rhizobial and agrobacterial plasmids are incompatible (Hynes et al., 1985; O'Connell et al.,

1987). Common repetitive and IS sequences do exist in rhizobia and agrobacteria (Krishnan and Pueppke, 1991; Deng et al., 1995).

2) Many plant reactions are common for development of nodules and for defense against pathogens, e.g., synthesis of flavonoids, callose, chitosans, proteinase inhibitors, chitinases (Werner et al., 1994; Phillips and Kapulnik, 1995; Spaink, 1995; Baron and Zambryski, 1995; Mellor and Collinge, 1995). The gene systems involved in the synthesis of exo(lipo)-polysaccharides and glucans in pathogenic bacteria (*Agrobacterium, Pseudomonas, Xanthomonas, Erwinia*) and in rhizobia are essential for the cross-talk with the plant defense systems (Leigh and Walker, 1994; Breedveld and Miller, 1994). However, the defense reactions in the normally developing nodules are expressed much more weakly than during the interactions of plants with the pathogens. The strong defense responses in the morphologically abnormal (empty) nodules are usually elicited by the rhizobia mutants defective in the synthesis of exopoly-saccharides or glucans (Niehaus et al., 1993; Heidstra and Bisseling, 1996).

The transient defense reactions are induced also during interaction of plants with endomycorrhizal fungi which are the most ancient and broadly distributed symbionts of vascular plants (Kapulnik et al., 1996; Gianinazzi-Pearson, 1997). It looks like the higher plants possess the universal systems for regulating the microbial interactions which may be either antagonistic or mutualistic as dependent on the symbiont genotype. These systems might have been evolved in ancient vascular plants when they colonized the land in close associations with fungi which ensured the water supply and mineral nutrition of their hosts (Malloch et al., 1980; LaRue and Weeden, 1994).

3) Gene-for-gene interactions play an important role in the legume nodulation. It is evidenced by the nodulation controlling gene-for-gene systems, e.g., *nodX-sym-2* for *R. leguminosarum* bv. *viceae – Pisum sativum* (Downie et al., 1994; Heidstra et al., 1994); *nodM, csn1 – rwt1* for *R. leguminosarum* bv. *trifolii – Trifolium subterraneum* (Lewis-Henderson and Djordjevic, 1991; de Boer et al., 1994); *nodH – Nnr* for *S. meliloti – Medicago sativa* (Caetano-Anolles and Gresshoff, 1992) symbioses. The involved rhizobia genes (*nodX, nodM, nodH*) control different host-specific modifications of the Nod factors (Long, 1996).

The apparent similarities with the phytopathogenic systems allowed several authors to consider the legume-rhizobia symbiosis as a "controlled disease" (C.L.Wilson, 1977), as a "refined parasitism" (Djordjevic et al., 1987) or as a result of a "sympathogenesis" process (Spaink, 1995). However, an immediate evolutionary relationship between the legume-rhizobia symbioses and pathogenic systems looks improbable because there are almost no homologues between rhizobia *nod* and agrobacteria *vir* genes. The genetic transformation of the host cells with bacterial DNA essential for the crown

gall or hairy root infections (Otten et al., 1992; Zupan and Zambryski, 1995) is not involved in the nodule development.

The agrobacteria and some rhizobia use a common strategy to exploit the host metabolism: induction of the *in planta* synthesis of specific compounds, opines, which are excreted outside the plant and are catabolized by the free-living bacteria (Murphy et al., 1995; Hong and Farrand, 1996). However, the involved genetic mechanisms are different because: (i) the opines in tumors are made by the plant cells transformed with agrobacterial T-DNA while in nodules the opines are synthesized by bacteroids; (ii) the rhizobial opines are derivatives of inositol while the agrobacterial opines are diverse compounds (derived from arginine, mannityl or chrizopine); (iii) for agrobacteria the synthesis/catabolism of opines is the major mechanism for exploiting the host while for rhizobia it is a minor mechanism (only 10–14% of virulent *S. meliloti* and *R. leguminosarum* strains possess this property). However, the opine metabolism may be an important factor of evolution of symbiotic N_2 fixation because it is correlated with nitrogenase synthesis in bacteroids (see p. 351).

Analysis of the taxonomic data (see p. 338) leads us to the assumption that the legume ancestors have been somehow predisposed for a coevolution with N_2-fixing eubacteria. Among the dicotyledonous plants an ability to form nodules with rhizobia is restricted to legumes, while nodulation by the N_2-fixing actinomycete *Frankia* is distributed in diverse families of the clide Rosid 1 (e.g., in Elaegnaceae, Rhamnaceae, Myricaceae, Casuarinaceae but amazingly not in Leguminosae) (Becking, 1970; Doyle and Doyle, 1997; Sprent, 1997). There are sufficient similarities in mechanisms of initiation of rhizobia- and *Frankia*-inhabited nodules (Pawlowski and Bisseling, 1996) suggesting that some general predisposition(s) for the subsequent evolution of the root symbioses with N_2-fixers appeared in a "common ancestor" of the Rosid 1 plants. The way in which this predisposition(s) were realized in different plant families might have been dependent greatly on the host defense systems because there are numerous plant pathogens among gram-negative eubacteria, but not among the gram-positive actinomycetes. Probably, there were some peculiarities in the defense systems which preadapted the ancient legumes for a fine differentiation between the potentially beneficial and deleterious eubacteria. A comparison of the defense reactions in *Parasponia* and in related non-nodulating Ulmaceae genera (e.g., *Trema*) may clarify the symbioses origin in Leguminosae.

There are several hypotheses supposing origination of the legume-rhizobia symbioses from the beneficial plant-bacteria interactions. For example, intimate symbioses were suggested to originate from associations of N_2-fixing bacteria with the outer root surfaces (Fig. 2A). Several stages of the symbiosis evolution have been supposed: occasional interactions of the free-living

bacteria with plant roots; unstable symbioses on the root surfaces; invasion of the root cortex by bacteria; specialization of the plant tissues (Parker, 1957). There are extensive data on tight associations between higher plants and free-living N_2-fixers, some of which (*Azospirillum, Acetobacter*) can invade the intercellular spaces of the root cortex (Boddey et al., 1995). The data demonstrating penetration of N_2-fixing bacteria into the root hairs and root cortex cells of non-nodulating legumes were published by Bryan et al. (1996).

Another hypothesis suggests that the legume-rhizobia symbioses might have evolved from interactions between the free-living diazotrophs and the plants which formed spontaneously the nodule-like structures (pseudonodules) on their roots (Fig. 2B). This hypothesis was first presented by Bonnier (1961) and supported later by the NAR (Nodulation in the Absence of *Rhizobium*) phenomenon in alfalfa and white clover (Truchet et al., 1989; Joshi et al., 1991; Blauenfeldt et al., 1994). The pseudonodulation that occurs in axenically grown plants, is controlled by chromosomal genes, and is cytologically similar to normal nodulation. Possibly, the pseudonodules were the organs for the starch storage in the ancient legumes and a rapid flow of C-compounds in and out of
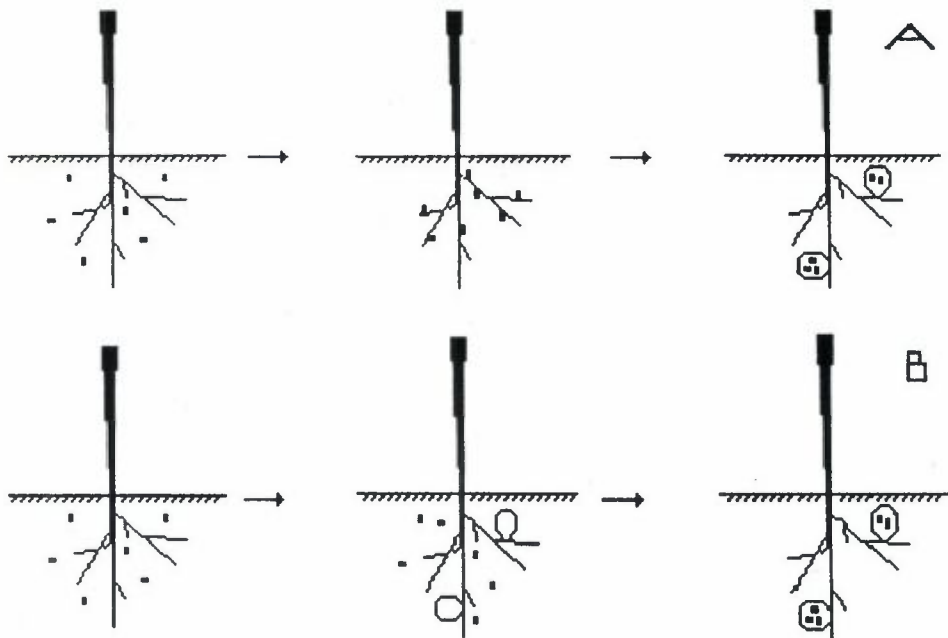

Figure 2.   Origin of the legume-rhizobia symbioses from associations of N_2-fixing bacteria with: A – outer root surfaces (Parker, 1957); B – spontaneously formed pseudonodules (Bonnier, 1961).

these organs predisposed the hosts for a metabolic integration with the endosymbionts (Caetano-Anolles et al., 1992).

To our mind, there is no great inconsistency between two proposed hypotheses (Fig. 2) because in the nodules of some "primitive" legumes, the rhizobia do not penetrate the plant cells (Sprent and Raven, 1992; Sprent et al., 1993). One can suggest that evolution of nodulation started from surface contacts between bacteria and plant cells (either inside the nodule-like structures or on the outer root surfaces) and was facilitated by the plant preadaptations for developing the nodule-like structures and for permitting a persistence of eubacteria inside them. An ability for symbiotrophic nitrogen nutrition (regulation and utilization of products of the *in planta* N_2 fixation) is possibly a more recent evolutionary gain of the plants which has been obtained independently by different taxa of the Leguminosae.

3. The genome plasticity and evolution of rhizobia

Recombination and transfer of sym genes

A striking feature of the rhizobia is their high genetic plasticity which is displayed as a frequent intragenomic recombination and a horizontal transfer of various genes. Unusually (for prokaryotes) numerous DNA repeats and IS elements constitute a basis for an intensive recombination in rhizobia (Martinez et al., 1990). Some symbiotic genes are represented by multiple copies, e.g., five *nodD* in *R. tropici* (Vlassak et al., 1995) and three *nifH* in *R. etli* (Romero et al., 1991). DNA sequences repeated 86-175 times were revealed in *Bradyrhizobium* (Minimisawa et al., 1995). Frequent deletions/amplifications of certain genome segments (amplicons) flanked by direct DNA repeats were detected in *R. etli* (Palacios et al., 1993, 1995). Various rearrangements (involving homologous and illegitimate recombination) are most frequent in those parts of the rhizobia genomes which contain the *sym* genes (Hahn and Hennecke, 1987; Kundig et al., 1993; Romero et al., 1995).

An involvement of the broad genomic rearrangements in the rhizobia evolution was first suggested by Heumann et al. (1984) who studied the alterations of colony types induced by the DNA damaging agents. It is attractive to consider that the genetic plasticity of bacteria was an important prerequisite for their coevolution with the angiosperms which are the swiftly evolving organisms with a high rate of genomic changes (Bobrova et al., 1985; Antonov, 1986; Kashin and Kuprianov, 1993). The rearrangements of the rhizobial *sym* genes may provide a synchronization of evolutionary rates in partners. As it was demonstrated for some parasitic systems, frequencies of mutations (per one generation) in coevolving organisms are comparable (Hafner

et al., 1994). Moreover, the increased mutation rates are characteristic for the organisms which are living under the periodically changing environment (including pathogenic bacteria) or which are conquering a novel ecological niche (Kimura, 1967; Ishii et al., 1989; Taddel et al., 1997). e.g., the mutant frequences are increased sufficiently among the *Agrobacterium* clones isolated from crown galls (Belanger et al., 1995).

A crucial role of the horizontal gene flow in the rhizobia **microevolution** is best illustrated by the data on intraspecies transfer of Sym plasmids in (*Sino*)*Rhizobium*. The basic facts are: (i) at least some Sym plasmids may be transferred due to their own *tra*-functions with frequencies up to 10^{-5} per recipient; (ii) an ability to form N_2-fixing nodules with the donor's hosts is often inherited after an intraspecies transfer of Sym plasmids (e.g., between different biovars of *R. leguminosarum*) (Beringer, 1982; Martinez et al., 1990); (iii) analysis of a linkage between the plasmid and chromosomal markers demonstrated an intensive pSym transfer in many populations of *R. leguminosarum* bv. *phaseoli*, *R. galegae* (Vlades and Pinero, 1992) and *R. leguminosarum* bv. *viceae* (Kinkle and Schmidt, 1991; Louvrier et al., 1996).

An input of the intraspecies transfer of Sym plasmids into the (*Sino*)*Rhizobium* microevolution may be restricted by their structural or functional incompatibility in different strains (Beynon et al., 1980; Espuny et al., 1992). However, this restriction may be surmounted via two mechanisms. First, an incoming Sym plasmid may substitute a resident one or recombine with it providing the novel bacterial genotypes (Christensen and Schubert, 1983; Wang et al., 1986). Second, the plasmid transfer may be facilitated by the asymbiotic (lacking Sym plasmids) strains present in the (*Sino*)*Rhizobium* populations (Soberon-Chavez and Najera, 1989; Segovia et al., 1991). The asymbiotic strains may arise from the symbiotic ones by a loss of *sym* genes (Sym plasmids) which are not essential for saprophytic survival of bacteria. These strains may restore the symbiotic properties after acquisition of Sym plasmids (Soberon-Chavez et al., 1991; Jarvis et al., 1989, 1995).

The asymbiotic strains which may acquire the nodulation ability after the *sym* gene transfer were isolated also from the populations of *R. loti*, a species which harbors its *sym* genes on the chromosome (Sullivan et al., 1995, 1996). These genes are located within the 400 kb chromosomal region, transfer of which between different *R. loti* strains may be facilitated by the flanking conservative insertion sites (Ronson et al., 1997; Sullivan and Ronson, 1997). However, a chromosomal location of *sym* genes may restrict sufficiently their horizontal transfer as it was demonstrated for *Bradyrhizobium* populations (Urtz and Elkan, 1996).

It is important to note that the plasmid transfer may be induced in rhizobia persisting in the legume nodules (Chernova et al., 1986; Pretorius-Guth et al.,

1990) or rhizosphere (Broughton et al., 1987). A similar induction was reported for some other plant-associated bacteria. For example, the plasmid transfer may be enhanced in the rhizosphere versus non-rhizosphere populations of *Pseudomonas fluorescens* (van Elsas et al., 1988). In *A. tumefaciens* a Ti plasmid transfer may be stimulated by octopine which is released from the plant tumors and activates the *tra* genes via the "quorum sensing mechanism" (Faqua and Winans, 1996). In *R. leguminosarum* this mechanism is involved in rhizospheral induction of *rhiABC* genes and in the regulation of the culture growth (Gray et al., 1996).

Involvement of a Sym plasmid transfer in the rhizobia **macroevolution** looks probable since acquisition of *sym* genes can impart an ability to nodulate the donor's hosts to: a) the rhizobia species genetically distant from the donor; b) various non-symbiotic bacteria which are either related to *(Sino)Rhizobium* species (*Agrobacterium, Phyllobacterium*) or are not related to them (*Pseudomonas, Escherichia, Lignobacter, Sphingobacterium*) (Hirsch et al., 1984; Plazinski and Rolfe, 1985; Van Veen et al., 1988; Novikova and Safronova, 1992; Fenton and Jarvis, 1994). These data suggest that *sym* genes may be rescued in bacterial communities if the climatic conditions are unfavorable for rhizobia survival. This rescue might have been of crucial evolutionary importance because the intensive movements into novel climatic zones occurred many times in course of legume evolution and domestication (Yakovlev, 1991). One can suppose that if the migrated hosts were accompanied by their microsymbionts, an origin of novel rhizobia types might have been induced. Novel microsymbionts might have evolved from soil bacteria which may persist in plant tissues without triggering a strong defense (e.g., the rhizobia nodulating some local legumes). A lack of correlation between rhizobial taxonomy and nodulation specificity (Fig. 1) may be the result of an intensive interspecies *sym* gene transfer in the course of symbiosis evolution.

The rhizobia evolution via a broad expansion of *sym* genes in soil microbe populations may be restricted by a lack of expression of *nif(fix)* genes in foreign genetic backgrounds. Nod[+] recombinants obtained after a Sym plasmid transfer between non-related rhizobia or to non-symbiotic bacteria usually cannot fix N_2 in symbiosis with the donor's hosts. Only low N_2 fixation was registered in the *Phaseolus* nodules formed by *Agrobacterium* harboring Sym plasmid from the broad-host-range *R. tropici* (Brom et al., 1988). However, this restriction may be surmounted by special selective mechanisms which ensure evolution of symbiotic N_2 fixation in rhizobia populations (see p. 350).

Bacteria-plant coevolution for nodulation

The sequence diversities of rhizobial nodulation genes do not correlate with the bacterial diversity but correlate with the taxonomy of the hosts, at least in the temperate legumes (Dobert et al., 1994; Ueda et al., 1995; Laguerre et al., 1996) suggesting a tightly coordinated bacteria-plant coevolution for nodulation. Based on the data on genetic control of rhizobial nodulation ability, its evolution could be imagined to involve several stages.

1) Formation of the system for synthesis of a core part of LCOs (*nodABCD* regulon) in free-living bacteria (diazotrophs, PGPR) associated with the roots of ancient legumes. The LCOs might be the effective nodulation stimulators because of their similarity with organogenic oligosaccharins or of their applicability for the cross-talk with the plant defense systems (Darvill et al., 1992; Spaink and Lugtenberg, 1994; Long, 1996).

2) Distribution of the *nodABCD* genes in the populations of soil (rhizospheral) bacteria via a horizontal transfer (Lindstrom et al., 1995a; Young and Haukka, 1996). It is supported by the aforesaid data on the highly conservative structure of *nodABCD* and on the lack of correlation between diversities of these genes and of 16S rDNA. The sequence analysis demonstrated that NodD proteins comprise a well-defined cluster in the family of LysR-like transcription regulators (Schlaman et al., 1992) supporting a common origin of *nodD* in taxonomically diverse rhizobia.

3) Formation of the gene systems for modification of LCOs in various rhizobia groups. The strain- and species-specific nodulation genes (e.g., *nodX*, *nodPQ*) responsible for a "non-allelic variation" of host specificity (Debelle et al., 1995) might have been evolved independently in different rhizobia. However, in evolution of an "allelic variation" of host specificity (when nodulation of a given plant is due to an allelic form of a given *nod* gene) a horizontal transfer of some nodulation genes between non-related rhizobia species might have been involved (e.g., transfer of *nodEF* between *S. meliloti* and *R. leguminosarum*).

Genomic instability (see p. 346) may have played an important role in the evolution of *nod* gene systems responsible for the production of LCOs. The LCOs are synthesized by the well-coordinated ensemble of rhizobial enzymes (transferases, transcription regulators, secretion proteins and others) which have homologues in non-symbiotic bacteria (Rhijn and Vanderleyden, 1995). In the majority of rhizobia the *nod* genes are grouped into several NodD-regulated operons which apparently evolved via a range of recombination events in ancestral organisms. The *Azorhizobium nodD* locus flanked by the repetitive elements (Geelen et al., 1995) might represent a vestige of these events. One can suggest that among the bacteria which interacted with roots of

ancient legumes the strains with a high genetic plasticity were the best candidates for transformation into rhizobia.

The selective advantages gained by bacteria from the nodulation gene evolution are obvious: penetration into the plants enables them to escape the environmental pressures and to grow using the host metabolites. However, the role of nodulation in the host survival might have been more complex. On one hand, close association with N_2 fixers enables the plants to grow under the combined N deficiency which had been especially severe in the times when legumes originated (Norris, 1956; Sprent, 1997). On the other hand, nodulation requires a sufficient loss of photosynthate and may increase a probability of plant penetration by various soil organisms. A biomass decrease was detected in the pea and soybean supernodulating mutants (Day et al., 1987; Rosendahl et al., 1989; Ohyama et al., 1993). Rhizobia inoculation may increase the legume susceptibility to *Phytophtora* (Gray and Hine, 1976), *Rhizoctonia* (Orellana et al., 1976) or *Glomus* (Xie et al., 1995).

These data suggest that in course of plant-bacteria coevolution the selective pressures operated which ensured formation of the mechanisms for restriction (regulation) of nodulation in legumes. At least two such mechanisms have been revealed: (i) a local one – LCO-degrading enzymes (e.g., chitinanses) which are induced in plant roots and can inactivate the Nod factors (Staehelin et al., 1995); (ii) a "systemic" one – the feedback regulation of nodulation in which the plant shoots are involved (Delves et al., 1986; Caetano-Anolles and Bauer, 1988; Gresshoff and Caetano-Anolles, 1992). The fate of a bacterial clone *in planta* is dependent on the host genes responsible for either organogenic or defense functions (Pawlowski and Bisseling, 1996; Long, 1996). The gene-for-gene mechanisms (Thompson and Burdon, 1992; Frank, 1992, 1994) might have been involved in evolution of the endosymbiont control in legumes. This type of coevolution is evidenced by occurrence of the gene-for-gene systems in legumes and rhizobia (see p. 342) and by their parallel variation in the centers of plant origin (Young and Matthews, 1982; Lie et al., 1987).

4. Why do rhizobia fix N_2 in nodules?

A facet of rhizobia research seeks to elucidate the mechanisms responsible for the evolution of symbiotic N_2 fixation because it is of direct ecological importance not for the bacteria but for the hosts. Only *Azorhizobium* strains (such as ORS571) were shown to be the true diazotrophs (Dreyfus et al., 1988). In (*Sino*)*Rhizobium* the N_2-fixing activity normally can not be induced *ex planta* while in *Bradyrhizobium* this activity, although possible in free-living bacteria, is not sufficient for supporting diazotrophic growth (Pagan et

al., 1975; Jordan, 1984). However, the numerous field experiments demonstrated that the number of rhizobia usually increases greatly in the soils occupied by the specific legume hosts, possibly due to the release from the nodules and/or by stimulation via the root exudates (Hirsch, 1996). An understanding of the selective mechanisms which ensured evolution of symbiotic N_2 fixation may give us a clue for the basic question of symbiosis evolution: "why should a symbiont benefit its host if it could gain immediate advantage by injuring it?" (Maynard Smith, 1989).

The kin selection (altruistic) mechanisms

A selection mechanism responsible for evolution of symbiotic N_2-fixation was suggested in the "altruistic model" (Jimenez and Casadesus, 1989). It assumes a preferential multiplication of Fix$^+$ bacterial clones *in planta* because the host ensures an intensive growth of Fix$^+$ nodules which than release more bacteria into soil than the Fix$^-$ nodules (Fig. 3A). The model is based on two versisimilar assumptions: (i) the Fix$^-$ nodules contain smaller amounts of bacterial cells and suffer earlier senescence and decay than the Fix$^+$ nodules; (ii) most nodules originate from the single bacterial cells, i.e., contain the clones; (iii) differentiated bacteroids are characterized by a decreased viability in comparison to non-differentiated cells. Therefore, differentiation of bacteria into N_2-fixing bacteroids was considered as a "sacrifice" of Fix$^+$ clone which permits its intensive propagation inside the nodules.

An attempt was made to extend the altruistic model on the interactions between legumes and rhizospheral rhizobia populations (Olivieri and Frank, 1994). A preferential multiplication of Fix$^+$ clones *ex planta* (Fig. 3B) was suggested due to action of some metabolites excreted specifically by those plants which have N_2-fixing nodules. The rhizospheral altruism may contribute significantly to the evolution of rhizobia which possess the genes for metabolism of the inositol-like compounds, rhizopines: *mos* genes for their synthesis induced in bacteroids and *moc* genes for their catabolism induced in free-living cells. The "rhizopine concept" assumes that the rhizobia strains which possess *mos/moc* genes are able to proliferate *ex planta* at the expense of other strains because the bacteroids transform the nodule metabolites into the selective growth substances to feed isogenic cells in the rhizosphere.

The "rhizopine concept" is consistent with the data that: (i) expression of the *mos* genes is controlled by the *nifA/ntrA* system, i.e., is coupled with the N_2-fixing activity; (ii) in *S. meliloti* and *R. leguminosarum* the rhizopine metabolism is encoded by Sym plasmids; (iii) the rhizopine catabolizing ability improves the nodulation competitiveness of rhizobia (Murphy et al., 1995; Wexler et al., 1996). However, a role of *mos/moc* genes in the maintenance
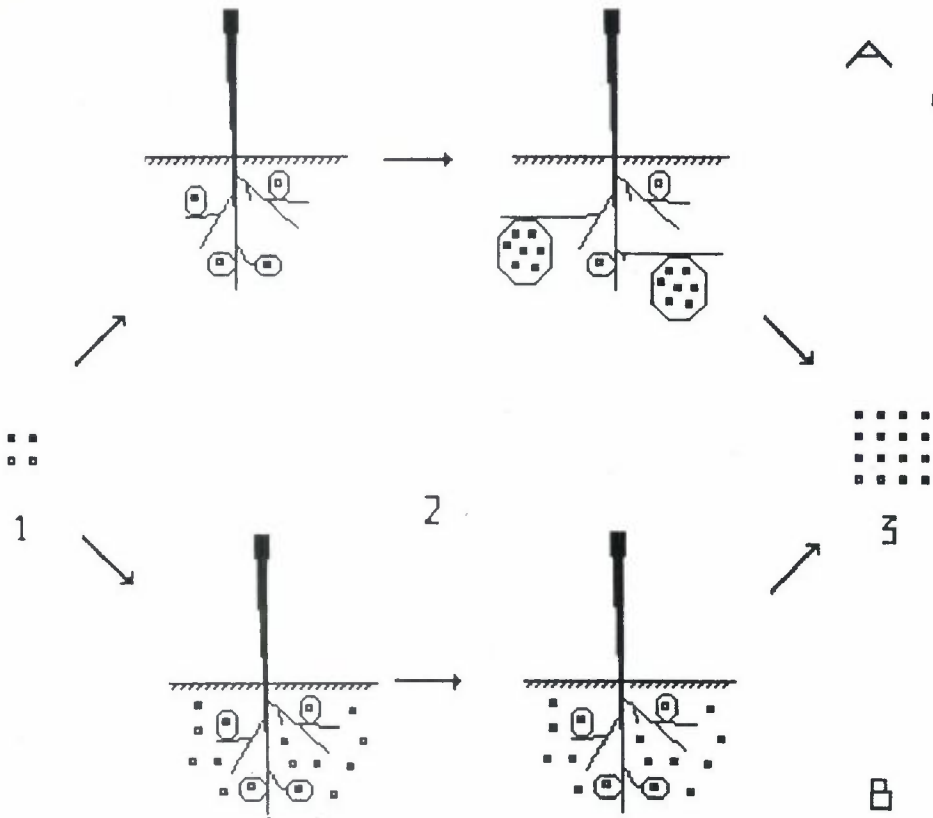
Figure 3. Altruistic models of evolution of symbiotic N_2 fixation. A – altruism in nodules
(Jimenez and Casadesus, 1989; simplified), B – altruism in the rhizosphere. Black
and white squares represent the Fix^+ and Fix^- clones in the bacterial
population: 1 – before, 2 – during, 3 – after its interaction with the host.

of rhizospheral rhizobia populations still has not been proven (Gordon et al.,
1996) and further analysis of an ecological significance of the rhizospheral
altruism is required.

Both models (Fig. 3) suggest that altruism is directed from one member of the
bacterial clone (highly differentiated N_2-fixing bacteroids) to the other
members (non-differentiated cells persisting in nodules or in rhizosphere).
Therefore, a mechanism similar to kin selection (Maynard Smith, 1964;
Darlington, 1978) is implicated in these models. The principal similarities are:
(i) altruism is of no or even of negative selective value for its donors but is
highly advantageous for its recipients; (ii) donors and recipients of altruism
are kin relatives. Involvement of kin selection may distinguish greatly the

mechanisms of partners' coevolution for symbiotic N_2 fixation and for nodulation because the gene-for-gene coevolution for nodulation (see p. 349) is based on the individual selection of Nod$^+$ bacterial clones.

Evolution of the legume-rhizobia symbiosis via the kin selection mechanisms is compatible with their involvement in: (i) transformation of the "severe" forms of parasitism towards the "benign" forms, i.e., in evolution of the antagonistic interactions towards the mutualistic ones (Pimentel, 1968); (ii) evolution of a high genomic plasticity (Kimura, 1967; Doolittle, 1982; Taddel et al., 1997) which apparently played an essential role in establishing the symbiotic system (see p. 346).

The individual selection and competition

The data suggesting involvement of kin selection in evolution of symbiotic N_2 fixation (see p. 351) do not exclude a possibility of an individual selection of Fix$^+$ bacterial clones. A mathematical modeling of the animal populations led to the "parsimony principle", which states a necessity to reject the kin selection if evolution of a given trait can be explained using an individual selection mechanism (D.S. Wilson, 1976, 1977). Several possibilities of evolution of symbiotic N_2 fixation via the individual selection of Fix$^+$ clones should be considered.

1) Symbiotic N_2 fixation may be a result of rhizobia adaptation to the stressful conditions (high acidity, low O_2 tension, plant defense reactions) inside the nodules (Udvardi and Kahn, 1992). Involvement of the stress-induced *groESL* genes in symbiotic N_2 fixation have been revealed in *S. meliloti* and *B. japonicum* (Fisher et al., 1994; Ogawa and Long, 1995). One can suppose that if symbiotic N_2 fixation is correlated to stress resistance, an individual selection of Fix$^+$ bacterial clones may be facilitated.

2) The hosts can select the rhizobia strains capable for intensive symbiotic N_2 fixation from the heterogeneous populations. For example, some alfalfa genotypes are preferentially infected by highly-active *S. meliloti* strains from a mixed population (Barnes et al., 1984). Some red clover tetraploids "prefer" to be infected by more active *R. leguminosarum* bv. *trifolii* strains than the parent diploids (Rubenchik et al., 1967). However, usually no correlation is revealed between the N_2 fixing activity of rhizobia strains and their potential to be "preferentially selected" by the host (Triplett and Sadowsky, 1992; Bottomley, 1992; Onishchuk and Simarov, 1995).

3) The evolution of mutualism may be analyzed using the Lotka-Volterra model which was developed initially for describing the competitive and predator-prey interactions (Travis and Post, 1979; Hallam, 1980). This model might be valid to analyze the dynamics of an idealized legume-rhizobia

system in which the mutualism is obligatory for both partners (Vandermeer and Boucher, 1978). However, the nodule occupation is a facultative stage of the rhizobia life cycle while the legumes can grow normally without rhizobia if combined N is available.

Moreover, there are data suggesting that the ability for effective symbiosis correlates with a decreased rhizobia survival *ex planta*: (i) numerous non-symbiotic clones were found in soil populations (Segovia et al., 1991; Jarvis et al., 1995); (ii) low-effective strains are prevailing in the major genetic classes (ET-types) while the highly-effective strains were found in the minor classes (Leung et al., 1994); (iii) a proportion of the effective strains is usually low in the populations persisting under stress (e.g., acid, high temperature) conditions (Jones and Burrows, 1969; Barber, 1980; Patel and Lambert, 1985; Graham, 1992; Fesenko et al., 1995); (iv) the symbiotic efficiency in rhizobia may be improved by deletions (losses) of some plasmids (Pankhurst et al., 1986; Jain and Bordeleau, 1990; Selbitschka and Lotz, 1991) or by $Tn5$ insertions into certain plasmid or chromosomal genes (Plazinski, 1981; Sharypova et al., 1994). In several *S. fredii* strains inactivation of *nolX* gene leads to the ability to form N_2-fixing nodules with those host genotypes, which even were not nodulated by the parent strains (Bellato et al., 1997).

These data suggest that the selective pressures do operate in rhizobia populations which decrease a number of clones capable for an intensive symbiotic N_2 fixation.   This decrease may be compensated via: (i) the aforesaid mechanisms of kin or individual selection of bacterial clones; (ii) a competition between Fix$^+$ and Fix$^-$ strains for the host nodulation. It was demonstrated (Amarger and Lobreau, 1982) that competition between two virulent strains (A and B) may be described by equation: $k_A/k_B = c\,(N_A/N_B)^a$ (k_A, k_B – numbers of nodules formed by two competing strains; N_A, N_B – concentrations of these strains in soil; "a", "c" – constant parameters). Analysis of different experimental systems demonstrated (Labandera and Vincent, 1975; Amarger and Lobreau, 1982) that typically the parameter "a" is lower than 1 (a = 0.2–0.8) while the parameter "c" may be either lower or higher than 1 (typically c = 1). One can see that if strain A is rare ($N_A < N_B$), the following inequality is fulfilled with the typical values of the parameters (a<1; c=1): $k_A/k_B > N_A/N_B$. This means that as a result of competition the rare strain A gains an additional opportunity to multiply *in planta*. For example, if a=0.2, a strain which constitutes only 10^{-5} of the mixed inoculum will form 10% of nodules.

We suggest this competition-for-nodulation mechanism to be of great evolutionary importance because it may ensure the multiplication of: (i) the Fix$^+$ strains if their survival in soil is decreased; (ii) the virulent recombinants which arise rarely after the *sym* gene transfer between different rhizobia

strains (species) or from rhizobia to non-symbiotic soil bacteria (see p. 346). Therefore, the evolutionary potential of rhizobia may be increased sufficiently due to their interactions with legume hosts.

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