CURRENT REVIEW

Bacteroid Development in Legume Nodules: Evolution of Mutual Benefit or of Sacrificial Victims?

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Symbiosomes are organelle-like structures in the cytoplasm of legume nodule cells which are composed of the special, nitrogen-fixing forms of rhizobia called bacteroids, the peribacteroid space and the enveloping peribacteroid membrane of plant origin. The formation of these symbiosomes requires a complex and coordinated interaction between the two partners during all stages of nodule development as any failure in the differentiation of either symbiotic partner, the bacterium or the plant cell prevents the subsequent transcriptional and developmental steps resulting in early senescence of the nodules. Certain legume hosts impose irreversible terminal differentiation onto bacteria. In the inverted repeat-lacking clade (IRLC) of legumes, host dominance is achieved by nodule-specific cysteine-rich peptides that resemble defensin-like antimicrobial peptides, the known effector molecules of animal and plant innate immunity. This article provides an overview on the bacteroid and symbiosome development including the terminal differentiation of bacteria in IRLC legumes as well as the bacterial and plant genes and proteins participating in these processes.

Symbiotic nitrogen-fixing bacteria, commonly termed rhizobia are α - or β -proteobacteria that establish symbiosis with members of the *Leguminosae* family and uniquely with the nonlegume genus *Parasponia*. The interactions between the bacteria and their host plant result in nodule formation, a novel plant organ developing usually on the root but occasionally also on the stem of the host plant. Nitrogen-fixing rhizobia called bacteroids are formed inside nodules in which, in exchange for photosynthetic products, they support plant growth by reduction of the atmospheric nitrogen gas into ammonia, which is then metabolized by the host plants. The development of nitrogen-fixing nodules is a complex multistep process that requires numerous signal exchanges between the partners (Gibson et al. 2008; Jones et al. 2007). In the majority of legumes, including in the model legumes *Medicago truncatula*

and *Lotus japonicus*, host specificity and nodule initiation depend on host flavonoid-induced production of Nod factors (NF) in the *Rhizobium* partner. The NF are signal molecules that trigger the initial steps of nodule development and are required for bacterial infection. NF perception and signal transduction pathways in the host root induce a number of biochemical and morphological changes (Oldroyd and Downie 2008). Cell division in the root cortex leads to the formation of the nodule primordium, while the NF-dependent infection mechanism implies formation of a tubular network of infection threads (IT), which is initiated in the root hair and then grows onward into the root cortex, transporting the bacteria to the nodule primordium. Although this sophisticated NF- and IT-dependent intracellular penetration of root hairs is the most common, NF- and IT-independent single-cell colonization through extracellular ‘crack-entry’ and formation of trans-cellular IT within the root cortex are used also for invasion of plant tissues (Held et al. 2010). These latter more ancestral infection strategies exist in extant legumes (Sprent 2007). Intriguingly, the *L. japonicus snf1* mutation affecting the NF perception in the root hairs results, occasionally, in the formation of functional nodules via NF-dependent crack entry or even NF- and IT-independent single cell infection (Madsen et al. 2010). In *Parasponia* spp., and certain legumes (e.g., some species of the *Papilionoideae* and many species of the *Caesalpinioideae* legume subfamilies), the bacteria remain in IT-like structures termed fixation threads, but in most nodulated plants, they invade the cytoplasmic space of the host cells via an endocytosis-like process releasing organelle-like structures called symbiosomes into the cytoplasm (Sprent 2007). The symbiosome is composed of either a single or of multiple bacterial cells surrounded with the peribacteroid space and a plant-derived peribacteroid membrane. The nodule initiation and infection processes as well as the known genetic determinants of these programs have been reviewed in great details by recent publications (Downie 2010; Gibson et al. 2008; Jones et al. 2007; Kouchi et al., 2010; Murray 2011; Oldroyd and Downie 2008; Popp and Ott 2011). This review focuses on bacteroid and symbiosome development with a special emphasis on terminal bacteroid differentiation and will discuss the bacterial and plant determinants that regulate this process.

DEVELOPMENT OF THE SYMBIOSOMES

The differentiation of symbiotic bacteria.

In the nodules containing infection threads (IT), bacteria reach the cells of the developing nodule through the IT net-

This review is dedicated to the memory of Adam Kondorosi. “He gleams like some departing meteor bright, Leaving behind him his own eternal light” (after Johann Wolfgang von Goethe). Adam Kondorosi leaves a legacy that will last for generations. The authors were privileged to be his coworkers through the years and E. Kondorosi to share a happy and unforgettable life with him.

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work. The IT is bordered by a membrane and cell-wall materials of plant origin and contain inside a matrix produced by both partners in which bacteria multiply (Brewin 2004). Although IT can grow topologically either inter- or intracellularly, bacteria are always in an extracellular space. The growth of the IT is driven by the bacteria that divide at the tip of the IT. Detailed ultrastructural studies describe individual steps of bacteroid development (Cermola et al. 2000; Szczyglowski et al. 1998; Vasse et al. 1990) in the two major (determinate and indeterminate) types of nodules (Crespi and Frugier 2008; Popp and Ott 2011). The determinate and indeterminate nodules differ primarily in the activity of the nodule meristem. In the determinate nodules, the meristem functions until the formation of the nodule primordium and the infected cells develop more or less synchronously to the nitrogen-fixation stage. In contrast, an active meristem persists in the indeterminate nodules, which produces constantly new generations of cells that can be infected by rhizobia and undergo a clear developmental gradient. Consequently, indeterminate nodules are composed of different nodule zones, which are the apical meristem (zone I), the invasion zone (zone II), the interzone II-III, the nitrogen-fixing zone (zone III), and the senescence zone (zone IV) in older nodules. The size of the meristem, zone II, and interzone II-III is constant, while zone III and IV increase with the nodule age. Commonly in both nodule types, the nitrogen-fixing symbiotic cells are huge plant cells that are generated with endoreduplication (ER) cycles, resulting in polyploid cells and cell expansion. In the indeterminate nodules, the ER cycles occur permanently in zone II and interzone II-III and are coupled to gradual increases of the nuclear and cell volumes. In both nodule types, the bacteria are released from the IT into the cytoplasm of the young nodule cells as infection droplets arising from unwallled tip regions of the IT. Inside the plant cytoplasm, the membrane of the infection droplet encloses bacteria that, after further biogenesis, forms the specific peribacteroid membrane (PBM). The bacterium and the surrounding PBM undergo division, followed by the formation of symbiotic compartments, which, depending on the host, contain either a single terminally differentiated bacteroid or multiple bacteroids via PBM fusion or further bacterial division. The size and morphology of bacteroids in those symbiosomes that contain multiple cells are similar to those of the free-living bacteria (Bisseling et al. 1977; Paau et al. 1979; Suganuma et al. 2003; Szczyglowski et al. 1998). Such symbiosomes are observed, for example, in *Lotus japonicus*, soybean (*Glycine max*), and bean (*Phaseolus vulgaris*). In contrast, in the indeterminate nodules of alfalfa (*Medicago sativa*), pea (*Pisum sativum*) or vetch (*Vicia sativa*), the bacteroids are strongly elongated and most often branched as well. These striking morphological alterations of the bacteroids compared with free-living bacteria were observed in vetch nodules as early as the 19th century (Beijerinck 1888). Vasse and associates (1990) described four stages of bacteroid maturation in alfalfa nodules. The newly-released Type 1 bacteria in the submeristematic cell layers are similar to the free-living bacteria, as they are rod-shaped and divide. The Type 2 bacteroids are nondividing and already elongated cells in the proximal region of the infection zone (zone II). In this zone, both the plant and bacterial cells grow. Type 3 bacteroids are already at their mature size (five to 10 times longer than the free-living cells) and occupy most of the host cell cytoplasm in the amyloplast-rich interzone II-III, in which conditions become microaerobic and the expression of the bacterial nitrogen-fixation genes (e.g., *nifA*, *fixK*, *nifH*) is sharply induced (Soupène et al. 1995). The Type 4 bacteroids are capable of nitrogen fixation that are distinguished from Type 3 bacteroids by their cytoplasmic heterogeneity and occupy the first eight to 12 cell layers of zone III.

The differentiation of bacteria involves dramatic changes in gene expression, cell surface structure, and metabolic activities that satisfy the demands of nitrogen fixation and are required for the adaptation of bacteria to the nodule cell environment. Plants maintain a very low and buffered free O₂ concentration within the fixation zone to prevent inactivation of the oxygen-sensitive nitrogenase enzyme complex (Ott et al. 2005). Rhizobia sense this low oxygen concentration with the help of the FixLJ two-component regulator system and adjust their transcriptome and metabolism to this environment (Fischer 1994; Prell and Poole 2006).

Formation of the peribacteroid membrane.

Infected cells contain tens of thousands of symbiosomes. Consequently, generation of symbiosomes necessitates an exceptionally extensive membrane synthesis in the infection zone that is about 30- to 50-fold higher than in other tissues (Robertson and Lyttleton 1984). The biogenesis of the PBM involves the fusion of the infection droplet membrane with ER- and Golgi-derived vesicles loaded with components of the peribacteroid membrane and space. While, initially, the uptake of bacteria by the plant cells was considered as an endocytotic process, later studies (Roth and Stacey 1989) as well as more recent investigations (Limpens et al. 2009) with the help of membrane identity markers revealed that symbiosomes are unique membrane compartments. Limpens and associates (2009) showed that early (SYP4) and late (Rab5) endosomal markers characteristic for the endocytotic pathway could not be detected in symbiosomes during their development. In contrast, the Rab7 late endosomal and vacuolar marker appeared on PBM after the symbiosomes stop dividing, while other vacuolar markers characteristic for lytic compartments (for example, SYP22 and VTI11) were absent until the senescence of the symbiosomes. Interestingly, the plasma membrane marker SYP132 appeared to label not only the plasma and IT membranes but also the PBM already from its release from IT up to the senescence stage (Catalano et al. 2007; Limpens et al. 2009). The concurrent presence of Rab7 and SYP132 on PBM confirmed its mosaic identity, which might allow both secreted and vacuolar-targeted plant products to be intercepted and used by the bacteroids and to be employed by the host to control microbial partners.

PBM is not only a barrier between the bacterium and the host to prevent a pathogenic interaction but, more importantly, it has a crucial role in the nutrient exchange between the symbiotic partners (Udvardi and Day 1997; White et al. 2007). Thus, it is not surprising that proteomics (Catalano et al. 2004; Panter et al. 2000; Saalbach et al. 2002; Wienkoop and Saalbach 2003) and biochemical investigations identified a number of transporter activities and proteins associated with PBM, such as a channel-like NH₄⁺ transporter (Tyerman et al. 1995), a dicarboxylate carrier (Ou Yang et al. 1990), the nodulin 26 aquaporin (Rivers et al. 1997), the anion-transporter nodulin N70 (Vincill et al. 2005), or Zn²⁺ (Moreau et al. 2002) and ferrous iron (Kaiser et al. 2003) transporters. In addition, a plant sulfate transporter targeted to the PBM proved to be essential for symbiotic nitrogen fixation (Krusell et al. 2005). The micronutrient borate is needed for cross-linking sugar residues in cell walls and glycoproteins as well as for symbiotic nitrogen fixation affecting multiple aspects of nodule development (Redondo-Nieto et al. 2008). Notably, it was shown that under borate starvation, symbiosome formation in pea nodules is arrested at a juvenile stage. Symbiosome proteins such as the lectin-like protein PsNLEC1 and the peribacteroid membrane-located rhamnogalacturonan II glycoproteins were not properly transported to the symbiotic compartment, suggesting that vesicle transport towards the symbiosomes is impaired under

boron starvation (Bolaños et al. 2001; Redondo-Nieto et al. 2007).

During PBM development, numerous proteins, including nodule-enhanced or nodule-specific gene products (called nodulins), are targeted to the symbiosome membrane and the peribacteroid space. The known proteins of the peribacteroid space are ENOD8 (Coque et al. 2008), a small family of calmodulin-like proteins (Liu et al. 2006), and the above mentioned PsNLEC1 lectin (Kardailsky et al. 1996). However, the biochemical or cellular functions of these proteins remain to be discovered. Transcriptome analysis of *M. truncatula* nodules revealed that more than 50% of the nodule-specific genes encode secretory proteins or peptides (Maunoury et al. 2010). This finding supports the assumption that many or most of the nodule-specific secreted proteins are targeted to the symbiosomes. Indeed, the importance of the secretory pathway for protein transport to symbiosomes was demonstrated by the recent discovery of the *M. truncatula* *MtDNFI* gene encoding a symbiosis-specific SPC22 subunit of the signal-peptidase complex (SPC) of the secretory pathway (Wang et al. 2010) activated in nodule zones II and III (Maunoury et al. 2010; Wang et al. 2010). A mutation in this gene arrested symbiosome and bacteroid development (Van de Velde et al. 2010; Wang et al. 2010) and blocked the transport of nodule-specific peptides to the symbiosomes (Van de Velde et al. 2010).

Genetic determinants of the symbiosome formation.

Genetic analysis of bacterial and plant mutants has been a very effective approach to dissect the initiation, development, and functioning of the symbiosis into elementary steps and to identify the genes and proteins concerned. The initiation of legume symbiotic interactions and the infection process have been in the focus of most studies that resulted in a detailed picture of the signals and their signal transduction pathways (Kouchi et al. 2010; Murray 2011; Popp and Ott 2011). In contrast, and despite the large number of available bacterial and plant mutants affected in the later steps of nodule development, only limited molecular data are available on symbiosome formation. A complex and coordinated interaction operates between the two partners during the maturation of symbiotic cells and the bacterial or plant mutants can provoke similar morphological, transcriptional, and developmental defects (Mittra and Long 2004).

It seems that any failure in the differentiation of bacteria into bacteroids prevents the subsequent transcriptional and developmental steps and can cause an incompatible interaction activating bacterial cell death and lysis (Werner et al. 1984) leading to early senescence of nodules. For example, a mutant in the *Rhizobium leguminosarum* bv. *viciae* *gltB* gene (encoding glutamate oxoglutarate amidotransferase involved in ammonium assimilation) is released from the infection threads, but the bacteria could not differentiate into bacteroids in pea nodules (Mulley et al. 2011). The mutation inhibited amino acid transport through the broad specificity amino acid transporters Aap and Bra by an undefined posttranscriptional mechanism that is likely triggered by an increased intracellular glutamine/glutamate ratio in the mutant. The defects in amino acid transport as well as in symbiosis could be rescued by second-site suppressor mutations in the gene coding for the RNA chaperone Hfq, which increased the expression of a number of ATP binding cassette (ABC) transporter genes, including *Aap* and *Bra*. Overexpression of plasmid-borne amino acid transporters such as Aap or Bra restored only the amino acid transport in the *gltB* mutant but did not rescue the symbiotic defect. In line with this, the *gltB* mutant exhibited a more severe phenotype than the *aap/bra* mutants (Lodwig et al. 2003), suggesting that, in addition to Aap and Bra, one or more other cru-

cial systems must be affected in the *gltB* mutant. This indicates that bacteroids need a supply of amino acids and other nutrients from the host cells for their early development. The transport of branched-chain amino acids through the Aap and Bra transporters is also important at later stages of bacteroid differentiation (Lodwig et al. 2003; Prell et al. 2009), as the branched-chain amino acid biosynthetic genes are down-regulated in the bacteroids, which, therefore, become symbiotic auxotrophs (Prell et al. 2009, 2010). Nevertheless, the requirement for these amino acids from the host cell must be rather low, since a partial *aap/bra* mutant with 10% transport activity was fully effective in symbiosis (Prell et al. 2010). Symbiotic auxotrophy of bacteroids occurs also in bean plants but was not observed in the *Sinorhizobium meliloti*-alfalfa symbiosis under the used conditions. Therefore, symbiotic auxotrophy may not be general in legume nodules or it may be a phenotype right on the edge, depending on subtle factors (Prell et al. 2010).

Type III secretion systems are flagella-derived molecular machines used by animal and plant pathogens to inject proteins (effectors) into the cytoplasm of host cells. Some but not all rhizobia have these protein secretion systems that affect the symbiosis in a positive or negative fashion, depending on the host plant (Deakin and Broughton 2009). In *Sinorhizobium fredii* NGR234, the absence of a type III effector named Y4IO results in the formation of defective symbiosomes (Yang et al. 2009). Y4IO is a putative acetyltransferase, however, its exact biochemical function and its one or more targets in the plant cells remain to be elucidated.

In *S. meliloti*, the *bacA* mutant (discussed in detail later) is released from the IT, but bacteroids fail to elongate and to form mature bacteroids (Glazebrook et al. 1993). *S. meliloti* wild-type and *fixG*, *fixJ*, or *nifH* mutant bacteroids isolated from *M. truncatula* nodules were morphologically similar (Maunoury et al. 2010). In contrast, the *S. meliloti* *fixLJ* mutants in alfalfa nodules were arrested as Type 2 bacteroids and only few cells of the *fixGH* mutants were able to differentiate into Type 3 bacteroids (Vasse et al. 1990). The expression of the *MtN31*, *MtCAM1*, and *MtLB1* host marker genes were more pronounced in the nitrogenase-deficient *nifD* mutant than in the *fixLJ* mutant. This indicates that the low oxygen concentration processes regulated by FixLJ are also involved in the maturation of bacteroids and that the host cell monitors bacteroid differentiation (Mittra and Long 2004).

A number of rhizobial mutants highlight the importance of the bacterial adaptation to the nodule and symbiosome environment. Mutations causing the truncation of the lipopolysaccharide (LPS) core (Campbell et al. 2002) in *S. meliloti* or the absence of the very long chain fatty acid (VLCFA) anchoring the lipid A to the outer membrane in *R. leguminosarum* bv. *viciae* and *S. meliloti* resulted in delayed and abnormally large and branched bacteroid formation, defective in nitrogen fixation and competitiveness (Haag et al. 2009; Vedam et al. 2004). In addition, strains lacking the correct LPS structure are more sensitive to cationic antimicrobial peptides (Campbell et al. 2002) or require more salt (most probably to maintain the stability of their outer membrane) for growth (Haag et al. 2011a) than wild-type bacteria. Thus, LPS mutants might be compromised in resistance to the specific environment (plant defense, radicals) in the PBM space (Campbell et al. 2002).

S. meliloti requires cobalamin (vitamin B₁₂) synthesis for symbiosis (Campbell et al. 2006). Ribonucleotide reductase (RNR) catalyzes the rate-limiting step of DNA synthesis, the conversion of ribonucleotides (NDP and NTP) into deoxynucleotides (dNDP, dNTP). *S. meliloti* uses a cobalamine-dependent RNR encoded by the *nrdJ* gene to support the intensive DNA replication during bacteroid development instead of the more commonly used cobalamine-independent enzyme

(Taga and Walker 2010). Expression of a heterologous, *Escherichia coli* cobalamine-independent RNR in the *S. meliloti* *nrdJ* mutant is sufficient for bacterial growth in the free-living state or in IT but fails to support terminal differentiation of the bacteroids, leading to rapid lysis of the bacteroids (Taga and Walker 2010). Cobalamine-independent and -dependent RNR display important differences in catalytic properties, and notably, the cobalamin-independent enzyme might be more sensitive to inactivation by reactive oxygen species that are abundantly produced by the host during infection of the nodules (Taga and Walker 2010). Indeed, *S. meliloti* bacteroids undergo a strong oxidative stress, as indicated by the activation of enzyme activities (catalase and superoxide dismutase) that detoxify reactive oxygen species (Santos et al. 2000; Sigaud et al. 1999) and by the aberrant bacteroid development in strains defective in oxidative stress protection (Davies and Walker 2007; Harrison et al. 2005; Jamet et al. 2003; Santos et al. 2000; Sigaud et al. 1999).

The nitrogen-fixing nodule cells in *M. truncatula* and *M. sativa* are polyploid (32 to 64C) and about 80-fold larger than the meristematic 2C and 4C cells (Cebolla et al. 1999). The repeated ER cycles and, thus, consecutive genome doublings in zone II are controlled by the cell-cycle switch gene *CCS52A* (Cebolla et al. 1999). Antisense-mediated downregulation of *CCS52A* in *M. truncatula*, resulting in smaller symbiotic cells with lower ploidy levels, aborted symbiotic cell and bacteroid differentiation (Vinardell et al. 2003). These observations indicate that formation of large polyploid symbiotic cells might be essential to maintain the high numbers of bacteroids and support them with nutrients and energy via the presumed increase of transcriptional and metabolic activities of the polyploid genome.

The EFD (ethylene response factor required for nodule differentiation) transcription factor is expressed in zone II and, besides inhibiting new nodule formation, it is also required for the differentiation of symbiotic cells (and the bacteria therein) by the inhibition of cytokinin signaling via the *MtRR4* response regulator (Vernie et al. 2008). In the *efd-1* mutant, not only the *MtRR4* gene but also several nodule-specific peptide coding genes (discussed later) were down-regulated.

With the help of morphological and transcriptome analysis of plant mutants, a temporal and spatial hierarchy of the genes and gene products were determined in the developmental program (Provorov et al. 2002). *SYMRK/DMI2/NORK*, *DMI3*, and *NSP1* genes are required for NF signaling in the very early steps of the interaction, before organogenesis and infection. However, hypomorph 'alleles' induced by RNAi or misexpression of the *SYMRK/DMI2/NORK* gene in a mutant background (Capoen et al. 2005; Limpens et al. 2005) or partial complementation of the knock-out alleles of the *DMI3* and *NSP1* genes with orthologous sequences from nonlegumes (Godfroy et al. 2006; Heckmann et al. 2006) usually allowed IT growth but failed to support the release of the bacteria into nodule cells. These phenotypes as well as the expression pattern (upregulation in the infection zone) of the genes coding for the elements of the NF signal transduction pathway (Bersoult et al. 2005; Indrasumunar et al. 2010; Popp and Ott 2011) suggest that a threshold level of NF perception is needed for the switch from IT growth to bacterial release. It is further strengthened by the observation that a mutation in *MtSYMREM1*, which codes for a nodule-specific remorin that interacts with *SYMRK/DMI2/NORK* and the NF receptors *LYK3* and *NFP* caused a very similar phenotype (Lefebvre et al. 2010). PBM development and bacterial release are also prevented in the pea *sym33* (Tsyganov et al. 1998) and the *L. japonicus alb1* (Imaizumi-Anraku et al. 1997) and *sym79* (Kawaguchi et al. 2002) mutants.

The LATD/NIP putative transporter of *M. truncatula* is indispensable for primary and lateral root formation and for nodule

development (Bright et al. 2005; Veereshlingam et al. 2004; Yendrek et al. 2010). In the mutants, the bacteria are either entrapped in abnormal and less numerous IT or, in the case of the least severe *nip-3* allele, a few of them are released into the cytoplasm, indicating again the need for the concerted action of nodule tissue development and rhizobial infection.

Numerous plant mutants have been isolated in which differentiation of the released rhizobia into bacteroids was inhibited (*Mtsym6* [Tirichine et al. 2000]; *Pssym26*, *Pssym27* and *Pssym32* [Morzhina et al. 2000]; *Ljsym75*, *Ljsym81* [Kawaguchi et al. 2002; Sukanuma et al. 2003]) or abnormal symbiosome structure was observed (*Pssym13* [Kneen et al. 1990], *Pssym31* [Borisov et al. 1997]). Interestingly, ultrastructural analysis of the *Pssym31* mutant revealed that the symbiosome protein PsNLE1 was produced but not properly targeted to the symbiosomes, suggesting that the secretory pathway to the symbiosomes is affected (Dahiya et al. 1998).

The *M. truncatula* defective in nitrogen fixation (*dnf*) mutants *dnf1*, *dnf2*, *dnf3*, *dnf4*, *dnf5*, *dnf6*, and *dnf7* were identified in a genetic screen for mutants forming nonfunctional nodules (Starker et al. 2006). Based on the expression of bacterial and plant symbiotic marker genes and nitrogenase activity, these seven mutants were classified in phenotypic groups (Mitra and Long 2004; Starker et al. 2006). In the *dnf1* mutant nodules, infected symbiotic plant cells were formed; however, the bacteroids did not differentiate and resembled free-living bacteria (Van de Velde et al. 2010; Wang et al. 2010). The other *dnf* mutants are expected to be arrested at later stages of symbiosome and bacteroid differentiation, although precise histological analyses of these mutants still need to be done.

Most rhizobial genomes lack the ortholog of the *nifV* gene of free-living diazotrophs encoding homocitrate synthase (HCS), an enzyme essential for the synthesis of the iron-molybdenum cofactor of nitrogenase and, therefore, for nitrogen fixation. Hakoyama and associates (2009) showed that in *L. japonicus* the homocitrate is provided by the host cell. HCS is encoded by the *FEN1* gene, which is exclusively expressed in infected cells and mutation in the gene abolished nitrogen fixation. Together with the above-mentioned symbiotic auxotrophy for branched-chain amino acids (Prell et al. 2009), this is another beautiful example of the metabolic dependence of bacteroids on their hosts and emphasizes that they behave like organelles.

AN EVOLUTIONARY TREND TOWARDS PLANT DOMINANCE

Host-dependent terminal differentiation of bacteria.

The above described morphological and cytological changes of bacteria in alfalfa nodules involve the elongation of the 1- to 2- μ m-long bacteria into bacteroids of 5 to 10 μ m in length that are packaged individually into PBM (Vasse et al. 1990). This increase is associated with a high level of genome amplification and the condensation of the genome content into multiple nucleoids of variable sizes (Bisseling et al. 1977; Mergaert et al. 2006; Paau et al. 1979; Vasse et al. 1990). This striking similarity to the host cell development, i.e., cell enlargement and polyploidization, and the observations that genetic and physiological interference with the bacterial cell cycle result in bacteroid-like structures (Cheng et al. 2007; Latch and Margolin 1997; Sibley et al. 2006; Wright et al. 1997) indicate that, during bacteroid development, the bacterial cell cycle is modified and multiple rounds of DNA replication take place without cytokinesis. The increased detergent sensitivity of the bacteroids (Sutton and Paterson 1980; Zhou et al. 1985) and the slow uptake of the membrane-integrity indicator dye propidium iodide (not taken up by intact cells) suggest that the integrity or per-

meability of the bacteroid membrane is affected (Mergaert et al. 2006). Since bacteroids isolated from these nodules cannot form colonies despite being metabolically active (Mergaert et al. 2006), it was concluded that bacteroids represent the end-point of an irreversible, thus, terminal differentiation program in *Medicago* nodules. All these bacteroid characteristics, i.e., enlarged size and DNA content, increased membrane permeability, and lack of reproductive ability, were also found in pea and vetch nodules but were not observed in the case of bacteroids from *L. japonicus* and bean nodules, indicating that the symbiotic rhizobia were not terminally differentiated (Mergaert et al. 2006).

Because the majority of our knowledge on the establishment of the symbiotic interaction has been based on the investigation of a few species (belonging to only three groups of the *Papilionoideae* subfamily) that are economically important (such as soybean, bean, alfalfa, pea) or were chosen as models (*M. truncatula*, *L. japonicus*), it was generalized that indeterminate nodule formation is associated with the development of enlarged, branched bacteroids while determinate nodules contain rhizobial cells that are similar in size and reproductive ability to the free-living bacteria. Several recent studies, however, changed this view. In the indeterminate nodules of two mimosoid legumes, *Mimosa pudica* and *Leucaena glauca*, bacteroids of the β -rhizobium *Cupravidius taiwanensis* and *Bradyrhizobium* sp. strain OK-79A, respectively, were not terminally differentiated (Ishihara et al. 2011; Marchetti et al. 2011). *Azorhizobium caulinodans* induces two types of nodules in symbiosis with *Sesbania rostrata* plants, indeterminate root nodules and determinate stem nodules. The comparison of free-living rhizobia and bacteroids from root and stem nodules revealed that independent of the nodule type, bacteroids were similar to cultured cells in size, morphology, DNA content, and membrane integrity (P. Mergaert, W. Van de Velde, M. Holsters, and E. Kondorosi, *unpublished results*). Phylogenetically, *Medicago*, pea, and vetch species are closely related and belong to the so-called inverted-repeat-lacking clade (IRLC) (known also as galeoid clade) of the *Papilionoideae* subfamily (Wojciechowski et al. 2004). Thus, these observations suggested that terminal bacteroid differentiation is not a general phenomenon of rhizobia; it is characteristic for the partners of IRLC legumes but independent of the nodule type.

To investigate whether the different fate of bacteroids in the different nodule types was due to differences in the bacterial genetic repertoires or to plant factors specific for IRLC legumes, Mergaert and associates (2006) compared nearly isogenic, recombinant rhizobial strains that were capable of establishing nitrogen-fixing symbioses with plants from different legume clades, such as pea and *Lotus japonicus* or vetch and bean. This comparison revealed that the host plant and not the genome of the bacteria determined the differentiation fate of bacteroids, because the same strain formed terminally differentiated bacteroids in the IRLC host and exhibited reversible differentiation in the non-IRLC legume. A similar dimorphism was observed in the case of rhizobial strain 32H1, which forms bacteroids like the free-living cells in cowpea but undergoes another unusual type of bacteroid differentiation in the *aeschynomeneoid* nodules of peanut, in which bacteroids are also enlarged but spherical (Sen et al. 1986). An extended investigation, using bacteroid morphology and DNA content as indicators of terminal or reversible differentiation, was carried out to place terminal differentiation into an evolutionary context (Oono et al. 2010). Mapping the bacteroid differentiation type on the legume phylogenetic tree and the reconstruction of the ancestral state revealed that the reversible fate is ancestral to the terminal one, which evolved independently in at least five clades of the *Papilionoideae* subfamily, suggesting convergent

evolution. It is very likely that each of these different legume lineages evolved their own special mechanism to impose terminal differentiation on their symbiotic partner.

Plant and bacterial factors in terminal differentiation.

The one or more genes responsible for the production of plant factors directing terminal bacteroid differentiation in the IRLC legumes were expected to be induced during nodule and bacteroid development, especially in the infected cells. Moreover, these plant factors were thought to be conserved in the IRLC legumes but absent in plants with reversible bacteroid differentiation. Nodule-specific cysteine-rich (NCR) peptides encoded by a gene family of more than 450 members in genus *Medicago* were identified as likely candidates with the analysis of the *M. truncatula* transcriptome and data mining in the public expressed sequence tag databases (Mergaert et al. 2003). The NCR genes are exclusively active in the nodule tissues and their expression is associated with symbiotic cell formation based on the transcriptome analysis of the symbiotic interactions between a large collection of mutant bacterial and plant lines arrested in different developmental stages (Maunoury et al. 2010). In situ hybridization or promoter- β -glucuronidase analysis revealed that the expression of all the tested NCR genes was restricted to infected cells, although in different expression domains (different cell layers or nodule zones), indicating their sequential induction and function (Mergaert et al. 2003). Homologous sequences were identified in other IRLC legumes such as vetch, pea, or *Galega*, but the sequenced genome of soybean and *Lotus japonicus* and the sequenced nodule transcriptome of *Phaseolus* species (harboring reversibly differentiated bacteroids) did not contain NCR genes (Alunni et al. 2007).

To prove that NCR peptides are the factors inducing terminal differentiation in rhizobia, different approaches were applied. The NCR genes code for short secreted peptides (of 25 to 50 amino acids in mature form) with two or three putative disulphide bridges, which resemble defensin antimicrobial peptides (AMP), the known effector molecules of animal and plant innate immunity (Maróti et al. 2011). With the combination of molecular (transgenic plants expressing NCR peptides fused to fluorescent proteins), immunological (immunofluorescence and immunogold localization), and biochemical (Western blot and mass spectrometry of the bacteroid proteome and peptidome, respectively) approaches, it was demonstrated that NCR peptides are directed to the bacteroids (Van de Velde et al. 2010). Targeting of NCR peptides to the symbiosome was dependent on the secretory pathway that is extremely prominent in symbiotic cells (Maunoury et al. 2010). The above-mentioned *dnf1* mutant of *M. truncatula* lacking the nodule-specific SPC made possible the study of the role of the secretory pathway in symbiosis. In this mutant, bacteroid differentiation was halted at an early phase (Van de Velde et al. 2010; Wang et al. 2010), as the signal peptide of the NCR peptides was not cleaved off. Therefore, the NCR peptides were not targeted to the bacteroids but remained blocked in the endoplasmic reticulum (Van de Velde et al. 2010). In a gain-of-function approach, *L. japonicus* plants, devoid of endogenous NCR genes and supporting reversible bacteroid differentiation, were transformed with NCR genes, which resulted in the appearance of features characteristic for terminal bacteroid differentiation, i.e., symbiosomes frequently containing only one, although a remarkably elongated, bacteroid (Van de Velde et al. 2010). Similarly, treating in vitro rhizobial cultures with pure NCR peptides induced responses reminiscent of bacteroid development, notably, an increase in membrane permeability, amplification of DNA content, cell elongation, and inhibition of cell division. Fluorescently labeled peptides accumulated in the cell envelope and some of them were then localized at the cell-

division plane coinciding with the inhibition of cytokinesis in the bacteroids, while others were found in the cytoplasm, in which they may interact with specific targets and affect cell functioning.

At present, the bacterial factors involved in the bacterial responses to the NCR peptides are unknown. However, the rhizobial *bacA* gene is a potential candidate. The *bacA* gene of *S. meliloti* is indispensable for the establishment of an effective symbiosis with alfalfa. The *bacA* mutant is able to induce nodule formation and is released from the IT into the plant cells but fails to further differentiate, which results in immediate senescence (Glazebrook et al. 1993) and in the cancellation of a transcriptome switch in the symbiotic plant cells associated with the terminal differentiation of bacteroids (Maunoury et al. 2010). The *bacA* gene is conserved in many bacteria and codes for an integral membrane protein belonging to the superfamily of ABC transporters. Under free-living conditions, pleiotropic phenotypes (reduced VLCFA content of the lipid A, increased sensitivity against detergents, low-level resistance towards the glycopeptide antibiotic bleomycin) associated with an altered cell envelope characterize the *bacA* mutant (Ferguson et al. 2002, 2004, 2005). In addition, the BacA protein facilitates the uptake of the Bac7 AMP, which is completely abolished in the mutant (Marlow et al. 2009). These phenotypes, however, cannot account on their own for the developmental defect of the mutant in symbiosis, because they can be uncoupled from the symbiotic phenotype, i.e., not all *bacA* mutants display the same phenotypic spectrum (LeVier and Walker 2001; Marlow et al. 2009). Strikingly, the requirement for BacA function in bacteroid development seems to correlate with the differentiation fate of rhizobia. While the *bacA* gene is indispensable for bacteroid development in rhizobia-nodulating IRLC legumes, *bacA* mutants develop into effective bacteroids in the indeterminate and determinate nodules of plants allowing reversible differentiation (Ardissone et al. 2011; Karunakaran et al. 2010; Maruya and Saeki 2010; Tan et al. 2009). Some of the NCR peptides have antimicrobial activity (Van de Velde et al. 2010), and the BacA protein provides resistance to these peptides (Haag et al. 2011b). The *bacA* mutant bacteria are challenged with NCR peptides in the nodule and die immediately after their release. However, they remain viable in the *M. truncatula* *dnf1* mutant, in which the NCR peptides are not transported to the bacterial compartment. Thus BacA seems to be critical to minimize the antimicrobial action of NCR peptides, thereby enabling bacteroid differentiation. Unfortunately, at present, no data are available on BacA from rhizobia undergoing terminal differentiation in the non-IRLC legumes (Oono et al. 2010). Thus, there is no indication whether terminal differentiation requires similar bacterial functions in different plant systems. Interestingly, BacA function is crucial for the pathogenicity of bacteria, such as *Brucella* or *Mycobacterium* spp., establishing chronic intracellular infection in macrophages (Domenech et al. 2009; LeVier et al. 2000). Since pathogens in macrophages also encounter AMP (Rosenberger et al. 2004), the presence of AMP seems to be the common feature of those chronic intracellular infections that require active BacA in the bacteria. This suggested that protection by BacA against host AMP may be conserved between symbiotic and pathogenic bacteria. BacA might facilitate the transport of peptides or, by affecting the composition and physico-chemical properties of the bacterial cell surface, it may influence the sensitivity of bacteria towards AMP.

Evolution of terminal differentiation.

At first sight, imposing a terminal differentiation on the endosymbionts might be a counter-productive strategy in an evolutionary context, because it could lead to the elimination

of the symbiotic bacteria and, therefore, to the extinction of the symbiotic interaction. However, the bacteria in a nodule represent generally a clonal population arising from a single colonizing *Rhizobium* cell. Besides the differentiated bacteroids, nodules also contain an important population of undifferentiated bacteria, for example, in infection threads or as saprophytic bacteria (Timmers et al. 2000). These subpopulations of undifferentiated bacteria make a significant increase in the bacterial population through the symbiosis and, therefore, constitute a sufficient advantage to the rhizobia to maintain the symbiotic trait (Oono et al. 2009).

Moreover, the multiple independent origins of host traits imposing terminal differentiation on the symbiotic partner (Oono et al. 2010) suggest an evolutionary advantage, i.e., the terminally differentiated bacteroids may provide more net benefits for legumes. Oono and Denison (2010) addressed this question by comparing the symbiotic efficiency (as measured by plant weight gained per nodule weight and nitrogenase activity per consumed carbon) of two rhizobial strains in symbiosis with both hosts allowing reversible differentiation (cowpea or bean) and host plants imposing terminal differentiation on them (spherical bacteroids in peanut or elongated bacteroids in pea). Though peas and peanuts are in distant clades and most probably have different traits and mechanisms to induce terminal differentiation, the terminally differentiated bacteroids in both pea and peanut were outperforming reversible bacteroids in bean and cowpea. In the case of the pea-bean comparison, an engineered strain was used that performed similarly to wild-type and natural symbiont strains of pea and bean, respectively (Oono and Denison 2010). Based on the analysis of these four symbiotic systems, it appears that terminal bacteroid differentiation may provide a selective advantage to the plant. Nevertheless, a more extensive comparison of symbiotic performance in legumes with terminal and reversible bacteroids is required to generalize this conclusion.

This result immediately raised the question why terminally differentiated bacteroids have higher symbiotic efficiency. There are multiple, nonexclusive explanations. It is well-documented in eukaryotic cells that enlarged polyploid cells have higher metabolic activity (Kondorosi and Kondorosi 2004). In addition, polyploid *Schizosaccharomyces pombe* cells are elongated and branched and are remarkably similar to terminally differentiated bacteroids (Fülöp et al. 2005). Thus, polyploidy might affect the eukaryotic and prokaryotic cells similarly, provoking cell growth and altered gene expression and metabolism of the cells. Moreover, multiple bacteroids in a symbiosome may have a more limited contact with the PBM and, therefore, a less-efficient nutrient exchange with the host than those symbiosomes in which only one bacteroid is enveloped by the PBM. It is also possible that, in the enlarged bacteroids, metabolic functions are partitioned in different areas of the cytoplasm (Young 2006), which increases symbiotic performance. In addition, the elongated bacteroids may be only one of the phenotypes, the most visible one, induced by the NCR peptides in IRLC legumes. The high diversity of NCR peptides that are produced in the nodules may indicate that these peptides have a broad spectrum of activities and can also modulate the metabolism of the bacteroids to improve their symbiotic performance. For example, the accumulation of the storage compound polyhydroxybutyrate (PHB), which diverts carbon supply from nitrogen fixation, is very frequently observed in reversibly differentiated bacteroids but not in bacteroids of the IRLC legumes. Inhibition of PHB accumulation could be a direct or indirect consequence of the terminal differentiation. Moreover, terminally differentiated bacteroids with their weakened membranes and locked-within nodule cells might be more effectively digested during nodule senes-

cence than reversible bacteroids. Thus, recycling their components might provide more nutrients for the plant. Taken together, the terminal bacteroid differentiation is potentially beneficial for the host in many different ways, thereby, the plant host dominates the interaction at the expense of the microsymbiont by imposing an altered physiology on it, which prevents life *ex planta*.

FUTURE DIRECTIONS

Although, in the last few years, a great deal of information has been collected on terminal bacteroid differentiation, the molecular mechanisms by which plants control bacteria are hardly known. A better understanding of terminal differentiation might allow us to modify other hosts to be similarly efficient. IRLC legumes use AMP-like molecules to control bacterial fate, but the effectors of other legumes remain to be identified. Certain NCR peptides affect the membrane integrity of bacteria and inhibit cell division, but the extreme structural divergence of the peptides suggests that other members of the family have additional functions as well. They might protect bacteria from the bactericidal effect of other NCR peptides or might directly affect transcription, translation, or metabolism, such as PHB synthesis. To address these questions, it will be essential to discover the molecular targets of individual NCR peptides and to identify those pathways that are affected in bacteroids. Another interesting topic is the precise mechanism by which BacA protein enables cells to succeed during chronic intracellular infection. A number of genomic tools in both symbiotic partners, including mutant and two-hybrid libraries, reference genomes for comparative transcriptome analysis, epigenetics, and mutation identification, as well as techniques of cell biology (e.g., microscopy, laser-capture microdissection) and biochemistry are needed and are in place to advance in this field.

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