Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*–legume symbiosis

Peter Mergaert*, Toshiki Uchiumi*†, Benoît Alunni*, Gwénaëlle Evanno‡, Angélique Cheron‡, Olivier Catrice*, Anne-Elisabeth Mausset*, Frédérique Barloy-Hubler‡, Francis Galibert‡, Adam Kondorosi*, and Eva Kondorosi*§

*Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Unité Propre de Recherche 2355, Avenue de la Terrasse Bâtiment 23, 91198 Gif-sur-Yvette Cedex, France; and ‡Unité Mixte de Recherche 6061, Bâtiment 13, Centre National de la Recherche Scientifique, Université de Rennes I, Faculté de Médecine, 2 Avenue du Pr. Léon Bernard, 35043 Rennes Cedex, France

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Symbiosis between legumes and *Rhizobium* bacteria leads to the formation of root nodules where bacteria in the infected plant cells are converted into nitrogen-fixing bacteroids. Nodules with a persistent meristem are indeterminate, whereas nodules without meristem are determinate. The symbiotic plant cells in both nodule types are polyploid because of several cycles of endoreduplication (genome replication without mitosis and cytokinesis) and grow consequently to extreme sizes. Here we demonstrate that differentiation of bacteroids in indeterminate nodules of *Medicago* and related legumes from the galegoid clade shows remarkable similarity to host cell differentiation. During bacteroid maturation, repeated DNA replication without cytokinesis results in extensive amplification of the entire bacterial genome and elongation of bacteria. This finding reveals a positive correlation in prokaryotes between DNA content and cell size, similar to that in eukaryotes. These polyploid bacteroids are metabolically functional but display increased membrane permeability and are nonviable, because they lose their ability to resume growth. In contrast, bacteroids in determinate nodules of the nongalegoid legumes lotus and bean are comparable to free-living bacteria in their genomic DNA content, cell size, and viability. Using recombinant *Rhizobium* strains nodulating both legume types, we show that bacteroid differentiation is controlled by the host plant. Plant factors present in nodules of galegoid legumes but absent from nodules of nongalegoid legumes block bacterial cell division and trigger endoreduplication cycles, thereby forcing the endosymbionts toward a terminally differentiated state. Hence, *Medicago* and related legumes have evolved a mechanism to dominate the symbiosis.

antimicrobial activity | bacteroid | endoreduplication | *Medicago* | nitrogen fixation

Symbiotic nitrogen fixation takes place in particular plant root organs named nodules. Nodule formation on plants of the Leguminosae family is a result of consecutive interactions with bacteria of the Rhizobiaceae family (rhizobia). The interaction is mutually beneficial. The bacteria within the nodule cells gain the ability to fix nitrogen gas by means of their nitrogenase enzyme complex and supply the host plant with the reduced nitrogen for plant growth. The plant provides photosynthates to the bacteria and a microaerobic niche for the oxygen-sensitive nitrogenase.

Nodule development is induced by lipochitooligosaccharide signals of rhizobia, called Nod factors. Nodules are formed on a particular host only in response to compatible rhizobia producing Nod factors with the adequate chemical structure (1). This is one of the major causes of the generally pronounced host specificity in *Rhizobium*–legume symbiosis. Nod factors induce cell divisions in the root cortex and successive divisions lead to the formation of the nodule primordium. Simultaneously, the rhizobia enter the host plant via the root hairs through the formation of tubular structures called infection threads which traverse the root epidermis and cortex and then the nodule primordium. Rhizobia are released from infection threads in the cytoplasm of postmitotic nondividing cells by endocytosis. The term "bacteroid" refers to these intracellular membrane-encapsulated bacteria. In legumes of the Papilionoideae subfamily, the nodules can be of either the determinate or the indeterminate type (2). In the case of determinate nodules, the initial cell division activity required for nodule primordium formation ceases rapidly and therefore the determinate nodules contain no meristem. Differentiation of infected cells occurs synchronously and the mature nodule contains symbiotic cells with a homogenous population of nitrogen-fixing bacteroids (2). Legumes such as bean (*Phaseolus vulgaris*) or *Lotus japonicus* form this type of nodules. In contrast, cell division activity in the indeterminate nodules is maintained and forms an apical meristem (nodule zone I). Because the size of the meristem is constant, cell division activity and production of new sets of meristematic cells are balanced with the exit of the same number of cells from the mitotic cell cycle. These postmitotic cells are unable to divide and enter the nodule differentiation program. The infection thread penetrates into the submeristematic cells and liberates the rhizobia. In the infected cells, both partners differentiate progressively along the 12–15 cell layers of the infection zone (or zone II), ending in the formation of nitrogen-fixing cells that will constitute the constantly growing nodule zone III (2). Legumes of the galegoid clade (such as *Medicago* spp., *Vicia sativa*, and *Pisum sativum*) are examples of plants forming indeterminate nodules.

In both nodule types, growth and differentiation of infected plant cells involve extreme cell enlargement. This cell enlargement is predominantly responsible for the growth of the nodule organ and is mediated by repeated endoreduplication cycles resulting in 64C or 128C polyploid nodule cells (C being the haploid DNA content) (3–8). The endoreduplication cycle is a modified cell cycle with replication of the genome (S phase) but without mitosis and cytokinesis (M phase). The polyploid state of a cell correlates generally with larger cell size, higher metabolic activity, and increased organelle content than the diploid one (3). In symbiotic nodule cells, high ploidy levels allow extreme cell growth, hosting thousands of bacteroids and sustaining the energy-demanding nitrogen fixation. Moreover, in many cell types endoreduplication is tightly linked to cell differentiation, and inhibition of endoreduplication results in developmental abnormalities. Reducing endoreduplication in nodule cells of transgenic *Medicago truncatula* plants by down-regulation of the *ccs52A* gene, a key regulator of the endoreduplication cycles, aborted nodule differentiation (7). The absence of nitrogen-fixing symbiotic cells in these nodules proved

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Abbreviations: CGH, comparative genomic hybridization; CTC, 5-cyano-2,3-di-4-tolyl tetrazolium chloride; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide.

†Present address: Department of Chemistry and BioScience, Faculty of Sciences, Kagoshima University, 1-21-35 Korimoto, Kagoshima 890-0065, Japan.

§To whom correspondence should be addressed. E-mail: eva.kondorosi@isv.cnrs-gif.fr.

that endoreduplication is an integral part of the symbiotic cell differentiation.

Interestingly, cytological studies showed that similarly to the hosting plant cells, the bacterial symbionts in the nodules of the galegoid legumes *Medicago sativa* (alfalfa) or *Vicia sativa* (vetch) undergo a profound differentiation process including important cell enlargement (9, 10). This finding raised the possibility that prokaryotes use the same strategy as eukaryotes, amplification of the genome, for differentiation and cell growth.

In this study, we show that differentiation of bacteroids in these legumes involves indeed genome amplification that is generated by endoreduplication cycles and correlates with elongation of bacteria. However, such bacteroid differentiation process is specific for galegoid legumes and absent from other legumes such as lotus and bean, where bacteroids are comparable to free-living bacteria. We provide evidence that bacteroid differentiation and endoreduplication are mediated by plant factors that are present in nodules of galegoid legumes but absent from nodules of other legumes.

Results

Genome Amplification of Bacteroids in the Indeterminate *M. truncatula* Nodules. More than 25 years ago, a 2- to 4-fold increase was estimated in the DNA content of *Sinorhizobium meliloti* bacteroids, the microsymbionts of *Medicago* species (11, 12). With the availability of more sensitive techniques we reinvestigated the relationship between cell size and genome size. *S. meliloti* bacteria and bacteroids, isolated from *M. truncatula* nodules, were stained with the fluorescent DNA dye 4′,6-diamidino-2-phenylindole (DAPI) and analyzed with Nomarski and fluorescence microscopy (Fig. 1*A*). The free-living cells were 1–2 μm long, whereas the bacteroids were 5–10 μm. Moreover, the bacteroids exhibited higher fluorescence corresponding to higher DNA content and were polynucleoid. The multiple nucleoids were in most cases randomly organized, with large cell-to-cell variations and differences in nucleoid sizes. The DNA content and size of cultured rhizobia and bacteroids were measured by flow cytometry (Fig. 1*B*). Compared with the 1C/2C DNA content of free-living *S. meliloti*, the DNA content of bacteroids peaked at 24C. Moreover, a positive correlation was found between the DNA content and the size of the bacteroids (Fig. 4, which is published as supporting information on the PNAS web site) similar to what is well established for eukaryotes (3).

In the literature (13) it is a long lasting controversy whether bacteroids are viable, able to resume growth outside the nodule. In our preparations, only 0.8% of the cells, likely arisen from undifferentiated rhizobia, formed colonies on agar plates, demonstrating that differentiated *S. meliloti* bacteroids are nondividing. To characterize better the physiology of bacteroids, we included the use of two fluorescent dyes, propidium iodide (PI) and 5-cyano-2,3-di-4-tolyl tetrazolium chloride (CTC) (Fig. 1*C*). PI, a frequently used DNA stain in viability tests, is excluded from living cells but enters cells with the loss of membrane integrity. As expected, PI did not color free-living, alive *S. meliloti*. In contrast, PI stained about 50% of bacteroids and all heat-killed bacteria and bacteroids. However, PI penetration was slow into the bacteroids whereas instant in the heat-killed cells. This indicated that the membrane integrity is slightly affected in the bacteroids but not comparable to that of dead bacteria or bacteroids. The increase in membrane permeability might be part of bacteroid differentiation required to facilitate the exchange of materials between the bacteroid and the host cell. CTC is an indicator of respiratory activity. This dye stained both the bacteria (96%) and bacteroids (97%) but not the heat-killed cells (0%). Therefore, *S. meliloti* bacteroids are alive, metabolically active cells, which, by being unable to reproduce, represent the endpoint of an irreversible differentiation program.

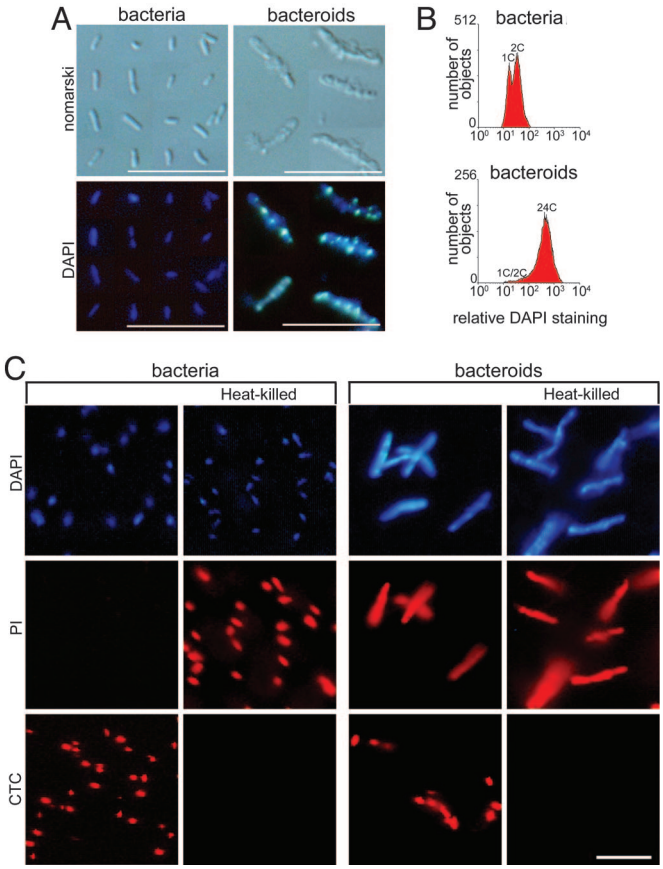

Fig. 1. Size, shape, and DNA content of free-living, cultured *S. meliloti* bacteria and *S. meliloti* bacteroids isolated from nitrogen-fixing *M. truncatula* nodules. (*A*) Nomarski (*Upper*) and fluorescence (*Lower*) microscopy of DAPI-stained bacteria and bacteroids. (*B*) DNA content of DAPI-stained bacteria and bacteroids measured by flow cytometry. (*C*) Fluorescence microscopy of bacteria and bacteroids stained with DAPI, propidium iodide (PI), or 5-cyano-2,3-di-4-tolyl tetrazolium chloride (CTC). ''Heat-killed'' indicates 10-min treatment at 70°C. (Scale bars, 10 μm.)

DNA Amplification in *S. meliloti* Bacteroids Involves the Whole Genome. The high DNA content in the *S. meliloti* bacteroids could arise from amplification of the entire tripartite genome, composed of the chromosome and two megaplasmids, pSymA and pSymB, or from amplification of particular regions in the genome. To distinguish among these possibilities, we compared the genomes of *S. meliloti* bacteroids and cultured *S. meliloti* with comparative genomic hybridization (CGH). The hybridization ratio of DNA from bacteroids and cultured bacteria of strain Sm1021 was close to 1 for all genes (Fig. 2*A*) as it was for the control comparing two samples of cultured Sm1021 bacteria (Fig. 2*B*), which indicated neither amplification nor deletion of specific regions in the bacteroid genome. To confirm this result, the sensitivity of CGH to detect genome alterations was tested in additional control experiments. The genomes of two wild-type *S. meliloti* isolates, Sm41 and Sm1021, differing in their geographical origin were compared, as well as the wild-type strain Sm41, with its symbiotically deficient deletion derivative ZB138, which carries a large deletion in pSymA encompassing the *nod–nif* region (14). CGH revealed significant differences between the genomes of the two wild-type strains (Fig. 2*C*). These differences were mostly detected in the two symbiotic plasmids. In ZB138, CGH revealed the known deletion as well as additional deletions in pSymA (Fig. 2*D*). Differences between the wild-type strains and deletions in ZB138 detected by CGH have

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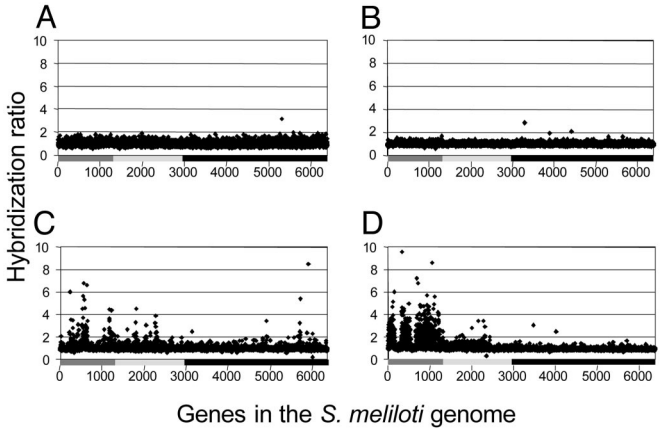

Fig. 2. Detection of genome changes in *S. meliloti* bacteria and bacteroids by CGH. (*A*) Comparison of bacteroids and cultured Sm1021. (*B*) Self-comparison of cultured Sm1021. The broader signal in *A* is due to higher experimental variability. (*C*) Comparison of *S. meliloti* strains 1021 and 41, differing in geographical origin but >99% similar at the nucleotide level. (*D*) Comparison of Sm41 and derivative ZB138. Each dot on the scatter plots is the Cye5/Cye3 hybridization ratio for the two compared samples (on the ordinate) for an individual gene (on the abscissa). A ratio of 1 indicates equal copy numbers in the two samples, whereas a ratio deviating from 1 indicates lower copy numbers or absence in one of the samples. The 6208 Sm1021 genes are ordered along the abscissa as they appear in pSymA (dark gray), pSymB (light gray), and chromosome (black).

also been proven by PCR (Fig. 5 and Table 1, which are published as supporting information on the PNAS web site). Taken together, these results demonstrated that CGH is appropriate to detect efficiently alterations in the genome. Because there was no difference between the bacteria and bacteroids by CGH, the 24C DNA content in *S. meliloti* bacteroids arose from endoreduplication of the whole genome.

Bacteroid Endoreduplication and Cell Enlargement Are Specific for Galegoid Legumes. Microscopic observations have shown that bacteroids in the indeterminate nodules of legumes closely related to *Medicago* are also enlarged (9). In *V. sativa* and *Pisum sativum* (pea) nodules, elongation of the bacteroids is coupled to branching resulting in a characteristic Y shape of the bacteroids (Fig. 3). Measuring the DNA content of free-living *Rhizobium leguminosarum* bv. *viciae* bacteria and bacteroids from *V. sativa* nodules revealed also a high increase (18C) in the DNA content of bacteroids (Fig. 3), which similarly to the *S. meliloti* bacteroids, were PI positive and did not form colonies on agar plates (0.4% of plated cells). We wondered whether these characteristics are general for all *Rhizobium*–legume symbiosis or specific for bacteroids in the indeterminate nodules of the galegoid legumes. To answer this question, we isolated and analyzed bacteroids from two symbiotic systems outside the galegoid clade and forming determinate nodules, which were the *P. vulgaris–R. leguminosarum* bv. *phaseoli* and the *L. japonicus–Mesorhizobium loti* interactions. Bacteroids in the bean or lotus nodules were indistinguishable from cultured rhizobia having the same morphology and DNA content, lacking PI staining (Fig. 3) and being able to form colonies (20%) on agar plates. Thus, the nitrogen-fixing form of these rhizobia was reversible in contrast to irreversible, terminal differentiation of bacteroids in galegoid legumes.

Plant Factors Provoke Bacteroid Differentiation in Galegoid Legumes. The different fate of bacteroids in the two nodule types could be due either to differences in the bacterial genetic repertoires or to plant factors specific for galegoid legumes. One could discriminate between these two alternatives if a bacterial strain were able to nodulate both a legume forming determinate nodules such as bean or lotus and a legume of the galegoid clade forming indeterminate nodules. To our knowledge, no known natural *Rhizobium* strain is able to do so. Nevertheless, some recombinant laboratory strains can cross this barrier. For example, *R. leguminosarum* bv. *viciae,* the microsymbiont of the galegoid legumes vetch and pea, was modified to nodulate *L. japonicus* (15). This recombinant strain, hereafter named *R. leguminosarum* bv. "Lotus," carries three additional nodulation genes, resulting in the synthesis of Nod factors that are recognized by *L. japonicus*. Another example is an *R. leguminosarum*

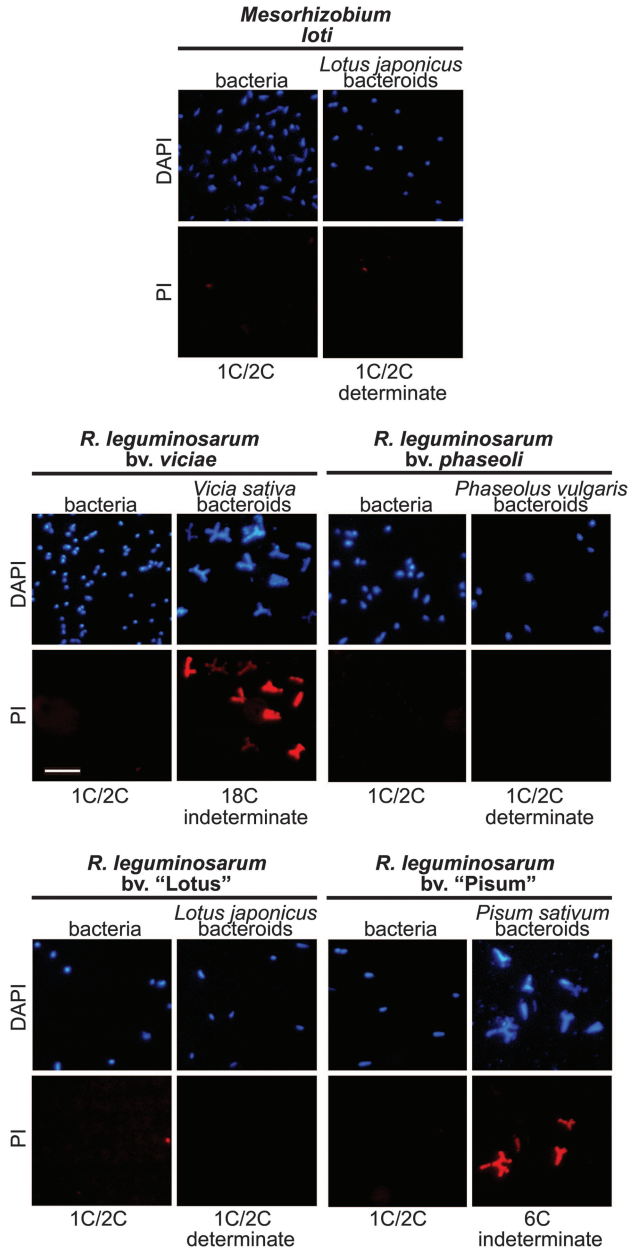

Fig. 3. Bacteroid differentiation is dissimilar in the indeterminate and determinate nodules and is controlled by the host plant. Bacteria and bacteroids for different symbiotic combinations were visualized by fluorescence microscopy after DAPI and PI staining. The DNA content is indicated below each sample as well as the nodule type from which bacteroids were purified. (Scale bar, 10 μm.)

bv. *phaseoli* derivative, which by carrying the symbiotic plasmid pRL1JI of *R. leguminosarum* bv. *viciae* became able to nodulate *Pisum sativum* (16). This strain is hereafter named *R. leguminosarum* bv. "Pisum." *R. leguminosarum* bv. "Lotus" bacteroids isolated from the determinate *L. japonicus* nodules were small, viable (26% colony growth), and PI impermeable, and they contained 1C/2C DNA. Therefore they have the same properties as bacteria in culture and as the *Mesorhizobium loti* bacteroids (Fig. 3). The *R. leguminosarum* bv. "Pisum" bacteroids in the indeterminate pea nodules were highly differentiated, displaying a strong increase in cell size and branching, DNA amplification, and PI staining (Fig. 3) similarly to the *R. leguminosarum* bv. *viciae* and not to the *R. leguminosarum* bv. *phaseoli* bacteroids. Moreover, they lost their capacity to resume growth when released from the nodules (0.4% colony growth). Thus, the same bacterial species can enter two entirely different differentiation processes to form nitrogen fixing bacteroids, being highly differentiated in nodules of galegoid legumes and visibly "undifferentiated" in lotus or bean nodules. These results show that bacteroid differentiation depends on the host plant. Thus, plant factors exist in the nodules of galegoid legumes, which control the bacterial cell cycle by inhibiting cytokinesis while allowing DNA replication, leading to endoreduplication, extreme cell growth, and increased membrane permeability.

Discussion

We have shown that the rhizobial bacteroids in nodules of galegoid legumes follow a completely different developmental path than bacteroids in nodules of nongalegoid legumes. The former undergo a profound transformation whereas the latter are much like free-living rhizobia for the characteristics analyzed. The transformations include an important elongation and in some cases also branching of the cells, an endoreduplication of the genome leading to multinucleoid cells, a permeabilization of the membrane, and finally, a loss of the ability to resume growth when released from the nodule. We also show that the terminal bacteroid differentiation in galegoid legumes is induced by plant factors and these plant factors are absent in nongalegoids. The influence of the host plant on bacteroid morphology has been reported previously for a distinct (aeschynomenoid) nodule type where bacteroids were spherical (17).

The correlation between cell size and genome multiplication is well established in eukaryotic cells (3). Our observations show that this correlation is also valid for bacteria. Few other examples in the bacterial world describe polyploid cells, but they all point to a similar correlation (18–20). Furthermore, it is remarkable that prokaryotic bacteroids and their eukaryotic host cells in the infection zone of indeterminate nodules follow similar differentiation paths proceeding synchronously and involving genome multiplication and cell enlargement (7, 10).

The bacteroids in galegoid legumes also showed a surprising resemblance to fission yeast undergoing endoreduplication cycles provoked by the premature degradation of mitotic cyclins. These polyploid cells are highly elongated, sometimes branched, and at a dead end of differentiation by loosing their cell division ability (21, 22). The viability of bacteroids (ability to resume growth and to produce descendants) is a long controversy in the literature (13). Our findings suggest that the loss of bacteroid viability in the galegoid legumes is related to the endoreduplication and multiple, apparently disorganized nucleoids in these cells, which may preclude performing cytokinesis correctly and producing mononucleoid cells with 1C DNA content. This is also impossible in endoreduplicated, highly polyploid eukaryotic cells. Moreover, the PI staining suggests that the membranes of the bacteroids of galegoid legumes became permeable for diffusion, which could also compromise the bacteroid's capacity to reproduce. Consequently, the recolonization of the rhizosphere at the end of the symbiosis, when nodules disintegrate by senescence, relies entirely on the nondifferentiated bacteria present in the infection threads in the infection zone of the indeterminate nodule (23). In contrast, the bacteroids in nodules of nongalegoids have none of these characteristics and therefore, they can produce offspring when released from the nodule. The absence of terminal bacteroid differentiation in determinate nodules is meaningful because terminal differentiation would imply no survival of the homogenous bacteroid population from a senescing nodule.

Studies on nodule development in general and bacteroid differentiation in particular are restricted to a few legumes of the estimated 12,000 legume species that are capable of symbiotic nitrogen fixation. Therefore, it has to be noted that our results obtained on galegoid legumes do not mean necessarily that indeterminate nodule development outside the galegoid clade also involves terminal bacteroid differentiation.

As we have shown in the example of determinate nodules, terminal bacteroid differentiation is not general for all *Rhizobium*–legume symbiosis and therefore it is not essential for bacteroid metabolism and nitrogen fixation. Then, what is the meaning of this differentiation process? Either the differentiated bacteroids have a better symbiotic performance, for example higher nitrogen fixation or better exchange of nutrients and fixed nitrogen, or, most intriguingly, the terminal bacteroid differentiation is a means by the plant to control proliferation of the bacterial endosymbiont. The advantage to the host could be multiple. A nodule is a very particular situation where the plant tolerates the presence of several millions of intracellular bacteria. Control on the bacterial proliferation can avoid spreading of rhizobia in tissues other than the nodule. It can also limit the release of rhizobia in the rhizosphere from senescing nodules, thereby moderating the impact of the symbiosis on the rhizosphere microflora. Moreover, the plant can recover nutrients from dying rhizobia during senescence, which is an obvious benefit for the plant. Indeed, in *Medicago* nodules, the cell content of bacteroids is entirely digested during nodule senescence (10) whereas in lotus nodules bacteroids largely survive nodule senescence (24). Whatever the advantage is to the host plant, the intentional limitation of bacterial viability puts a particular light on the symbiosis where instead of equality the plant has evolved a mechanism to dominate the symbiosis.

Plant factors are responsible for the bacteroid differentiation in galegoid legumes. However, also bacterial functions are essential for proper bacteroid differentiation in galegoid legumes. The BacA protein of *S. meliloti* is a peptide importer, localized in the bacterial inner membrane. Mutants in the *S. meliloti bacA* gene form nonfunctional nodules on *Medicago* spp. as the bacteria after their release from the infection threads undergo immediate senescence (25). In agreement with this observation, we observed that bacteroids of the *bacA* mutant are not elongated and do not amplify their genome (data not shown). Thus the BacA protein may be involved in the recognition or transport of the plant factors (peptides) for bacteroid differentiation. The premature senescence of $bacA^-$ bacteroids also implies that the plant senses the differentiation process and stops the interaction when the bacterium fails to differentiate.

The *bacA* gene is well conserved among bacteria, including nonsymbiotic bacteria. Therefore, this gene does not seem to be evolved specifically for the symbiosis and may have other functions. The gene is also well conserved in *Mesorhizobium loti*. Because differentiation by endoreduplication and cell elongation does not take place in *M. loti* bacteroids, it was expected that BacA is dispensable for *M. loti* bacteroids. Indeed, this is the case as nodulation and nitrogen fixation are not affected by the *bacA* mutation in *M. loti* (J. Maruya and K. Saeki, personal communication).

What could be the factors that galegoid legumes produce to inhibit rhizobial cytokinesis and to induce endoreduplication

and the typical bacteroid morphology? To help answer this question, an interesting parallel can be drawn between the endosymbiotic bacteroids and intracellular animal pathogens. Antimicrobial peptides (defensins) are part of the innate immune system and are activated in response to such pathogens. They provoke membrane permeabilization and inhibition of septum formation (26). For example, the assault of intracellular *Salmonella* with defensins results in inhibition of cell division and the formation of elongated cells (27). The lipopolysaccharides (LPS) of the pathogens are important virulence factors to overcome innate host defense mechanisms. LPS mutants of *Salmonella* or *Pseudomonas* species have an increased sensitivity to defensins, which is accompanied by a loss of intracellular survival and thus strongly reduced virulence of the strains (27, 28). In addition, the intracellular settling of pathogens involves structural modifications of LPS in such a way that the pathogen becomes more resistant to host antimicrobial peptides.

Interestingly, the rhizobial LPS also affects the bacteroid differentiation in galegoid legumes. Bacteroids of LPS mutants are abnormal (29–31). The *lpsB* mutant of *S. meliloti* was found to be more sensitive to antimicrobial peptides (30). Moreover, rhizobia modify their LPS structure during bacteroid differentiation in *Pisum* or *Medicago* nodules (32, 33), which could be an attempt to counteract plant defensin-like peptides. Taking together the above considerations, the plant factors involved in the terminal bacteroid differentiation in galegoid legumes could be defensin-like peptides. The recently described nodule-specific cysteine-rich (NCR) peptides that are present only in the infected nodule cells of galegoid legumes and are absent from other legumes and that share several characteristics with defensins are potential plant signals that may trigger bacteroid differentiation in coordination with the host cell (34). Whatever the nature of the plant factors, a similar eukaryotic control on bacterial cell cycle may also be relevant for pathogenic interactions, because rhizobia are closely related to plant, animal and human pathogens such as *Brucella*, *Burkholderia*, and *Ralstonia*.

Materials and Methods

Plants and Bacteria. Plant lines were *Medicago truncatula* R108, *Vicia sativa* subsp. *nigra*, *Pisum sativum* cv. Primdor, *Lotus japonicus* Myakojima MG20, and *Phaseolus vulgaris* cv. Tendercrop. Surface-sterilized seeds were germinated and planted on perlite substrate watered with a nitrogen-poor nutrient solution (34). After 1 week of growth, plants were inoculated with an appropriate rhizobial suspension (OD_{600} = 0.1). Nodules were harvested 3 weeks after inoculation.

Rhizobial strains used were for *S. meliloti*, strains Sm1021 (35) and Sm41 and ZB138 (14); for *R. leguminosarum*, strains RBL5560 (bv. *viciae*) and RBL5560.pMP2469.pMP2470 (bv. "Lotus") (15), strains 4292 (bv. *phaseoli*) (36), and A34 (bv. "Pisum") (16); and for *M. loti*, strain MAFF303099. Rhizobia were grown on TA (*S. meliloti* strains) (1% tryptone/0.1% yeast extract/0.5% NaCl/0.2% $MgSO_4 \cdot 7H_2O$/0.03%, $CaCl_2 \cdot 2H_2O$), YEB (*M. loti*) (0.5% beef extract/0.1% yeast extract/0.5% peptone/0.5% sucrose/0.04% $MgSO_4 \cdot 7H_2O$, pH 7.5), or TY (*R. leguminosarum* strains and *R. phaseoli*) (0.5% tryptone/0.3% yeast extract/0.05% $CaCl_2 \cdot 2H_2O$) supplemented with the appropriate antibiotics.

Bacteroid Characterization. Bacteroid isolation was performed as described in ref. 37. Bacteroids and free-living bacteria were stained with DAPI at 50 μg/ml, PI at 2 μg/ml, and CTC at 1.2 mg/ml and observed with a Reichert Polyvar fluorescence microscope connected to a Nikon dxm 1200 digital camera. DNA measurements were performed with a Beckman-Coulter ELITE ESP flow cytometer. For determination of the colony-forming units, 10^5 cells counted by flow cytometry and their dilution series were plated on selective medium.

CGH. For labeling of genomic DNA, 1 μg of MboI-digested DNA was denatured for 5 min at 94°C followed by the addition of 9 μg of random primers (Invitrogen), 1× NEB3 buffer (New England Biolabs), 0.1 mg/ml BSA, 20 mM DTT, 10 units of the Klenow fragment of DNA polymerase (New England Biolabs), 40 μmol each of dATP, dTTP, and dGTP, 25 μmol of dCTP; 1.5 nmol of Cye dye-labeled dCTP (Amersham Pharmacia Biosciences), and water to a final volume of 100 μl. After an incubation of 90 min at 37°C, the mixture was purified with the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). Cye5- and Cye3-labeled samples were pooled, concentrated, and resuspended in 50 μl of hybridization buffer (Nexterion, Schott, Louisville, KY) containing 10 μg of sonicated salmon sperm DNA (Invitrogen), then denatured 2 min at 100°C and loaded onto a *S. meliloti* Sm1021 whole genome oligoarray (Operon, Bielefeld University, Bielefeld, Germany) in a hybridization cassette (Telechem, Hybaid, Middlesex, U.K.). The arrays carry 6,208 70-mer oligonucleotides directed against the predicted protein-coding ORFs. Hybridization was at 55°C for 16 h and the washing steps were 5 min in 2× SSC/0.2% SDS at 55°C, 1 min in 0.2× SSC/0.1% SDS at 21°C (twice), 1 min in 0.2× SSC at 21°C (twice), and 1 min in 0.1× SSC at 18°C. Slides were dried by centrifugation. Analysis of hybridization signals was performed by using an Axon ScanArray 4000B scanner and the GENEPIX 5.1 PRO software (Molecular Devices, Sunnyvale, CA).

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