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Cupriavidus taiwanensis Bacteroids in *Mimosa pudica* Indeterminate Nodules Are Not Terminally Differentiated▽

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The beta-rhizobium *Cupriavidus taiwanensis* forms indeterminate nodules on *Mimosa pudica*. *C. taiwanensis* bacteroids resemble free-living bacteria in terms of genomic DNA content, cell size, membrane permeability, and viability, in contrast to bacteroids in indeterminate nodules of the galegoid clade. Bacteroid differentiation is thus unrelated to nodule ontogeny.

Bacteria known as rhizobia cooperate with legumes in a mutualistic endosymbiosis of major ecological importance that accounts for about 25% of the global nitrogen cycling. Rhizobia induce the formation of root nodules on host plants, in which intracellular bacteria fix nitrogen for the benefit of the plant (2). Diversity characterizes the rhizobium-legume symbiosis. This symbiosis involves most of the 18,000 legume species of the Papilionoideae, Mimosoideae, and Caesalpinioideae subfamilies. Rhizobia are phylogenetically disparate bacteria distributed in many genera of the alpha- and betaproteobacteria (5, 10). In addition, many phenotypic variations regarding the localization, shape, and anatomy of the nodules, as well as the infection mode and differentiation status of endosymbionts, called bacteroids, are encountered in nature (8).

Two types of nodules have been defined according to their ontogeny and development (6). Indeterminate nodules originate with dividing inner cortical cells and develop a persistent meristem at the distal end. Mature indeterminate nodules are characterized by a longitudinal gradient of plant cells and bacteroids at different stages of differentiation. Five steps in bacteroid differentiation have been defined, each being restricted to a well-defined histological region of the nodule (16). Determinate nodules instead originate with external cortical cells and differentiate in a synchronous manner, resulting in mature nodules that contain a homogenous population of nitrogen-fixing bacteria. It was shown that in indeterminate nodules of *Medicago* and related legumes of the galegoid clade, bacteroids of the nitrogen-fixing zone are terminally differentiated. They undergo profound cellular changes, including a size increase (mean, 5×), DNA amplification (mean, 24C), and modification in membrane permeability, and lose their capacity for reproduction (<1%) (9). In contrast, bacteroids of determinate nodules of *Phaseolus*, *Lotus*, or soybean differ little from free-living bacteria (9). Both histological types of nodules and the bacteroid differentiation level are controlled by the legume host (9, 11). It is so far unclear, however, whether nodule ontogeny and bacteroid differentiation are truly correlated.

We investigated this issue with an atypical model system. The beta-rhizobium *Cupriavidus* (formerly *Ralstonia*) *taiwanensis* nodulates *Mimosa pudica* (1, 4), a plant of the *Mimosoideae* subfamily that forms indeterminate nodules (3). Like most rhizobia, *C. taiwanensis* penetrates root tissues via root hairs from which infection threads elongate toward the emerging nodule. Bacteroids are released from infection threads in the cytoplasm of nodule cells, where they are enclosed in a symbiotic structure called the symbiosome. So far, the extent of bacteroid differentiation in *M. pudica* nodules has not been investigated.

We confirmed that under our experimental conditions, *M. pudica* formed indeterminate nodules. Seedlings of *M. pudica* were grown in Gibson tubes under N-free conditions and inoculated with 10^7 bacterial cells grown in TY as previously described (7) except that Gibson tubes contained only quarter-strength liquid Jensen. Either of the following *C. taiwanensis* LMG19424 derivatives was used as an inoculum: strain 204, which constitutively expresses *gfp* (3); strain CBM132, which contains a plasmidic *nodB-lacZ* fusion (7); strain CBM722, which contains the pCBM39 plasmid harboring a *nifH-lacZ* fusion; and strain CBM2153, which contains the pCBM78 plasmid harboring a *nifH-gfp* fusion. To construct pCBM39, the *nifH* promoter of *C. taiwanensis* was amplified using 5′-CCCA AGCTTGTTAGTTGCCAAGCGACGTA-3′ and 5′-TGCAC TGCAGCCATTTTGAATTGAAGGTGTAGC-3′ as primers and cloned into the HindIII-PstI restriction sites of pCZ388 (7). To construct pCBM78, the *gfp* gene was amplified using 5′-TGCACTGCAGTATAGGGAGACCACA-3′ and 5′-TG CACTGCAGCAGCAGCCAACTCAGC-3′ as primers and cloned at the PstI site of pCBM39 downstream the *nifH* promoter. Plants were harvested at different time points after inoculation and examined using confocal laser microscopy and light microscopy as described previously (7). Nodules emerged from the inner cortex (data not shown). At ca. 14 days post-inoculation (dpi), nodules harbored a distinct meristem at the tip of the nodules (Fig. 1A) that persisted at 35 dpi. In addition, mature nodules contained an invasion zone where cells were invaded by infection threads (Fig. 1B and D) and a fixation zone where nodule cells were massively infected by

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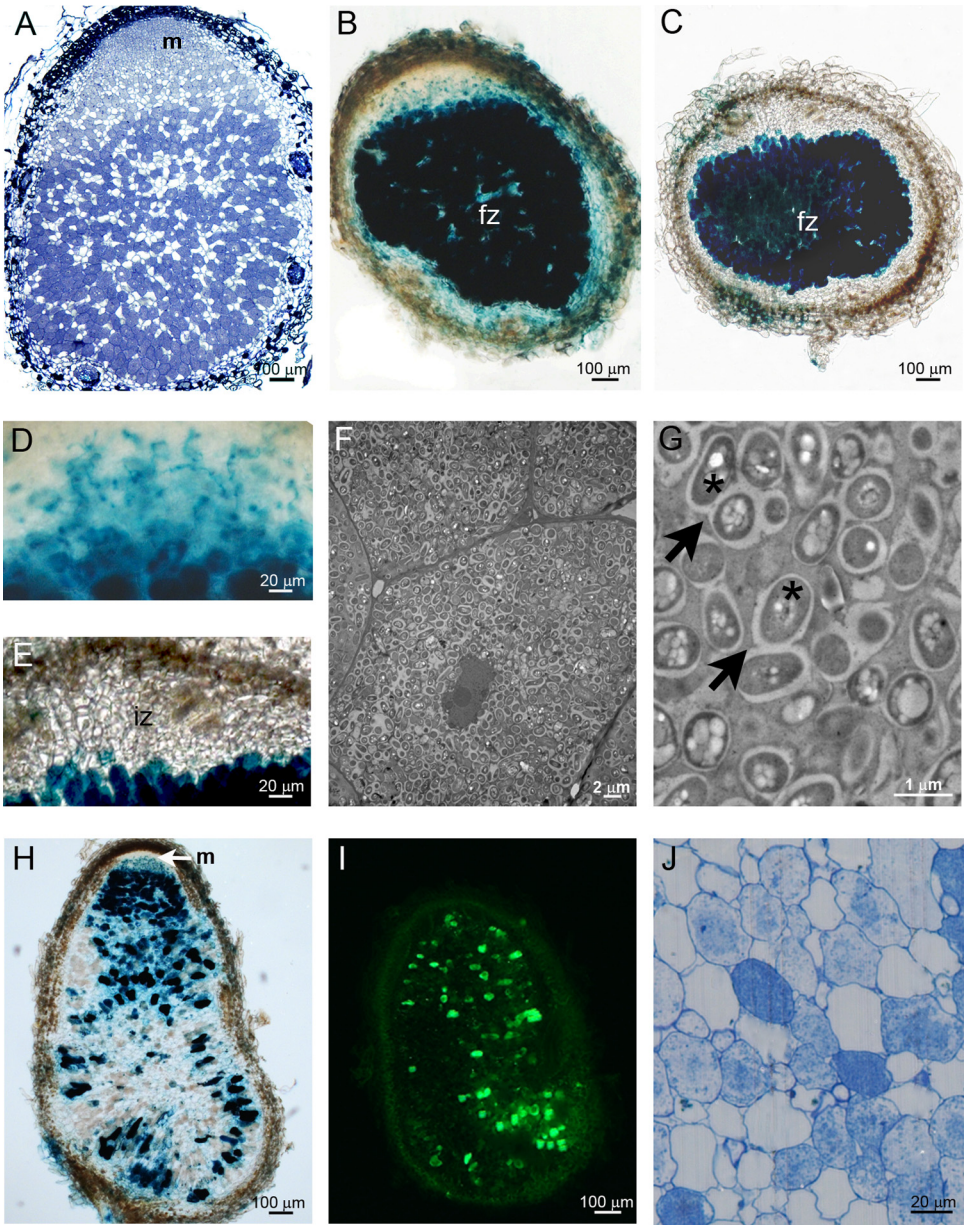

FIG. 1. Imaging of indeterminate nodules formed by *C. taiwanensis*: fluorescence (I), light (A, B, C, D, E, H, and J), and electron (F and G) microscopy of nodules formed by *C. taiwanensis* genomic GFP (A, I, F, and G), plasmidic *nodB-lacZ* (B, D, and H), or plasmidic *nifH-lacZ* (C and E) or stained with toluidine blue (A and J). An apical meristem was present in nodules formed at 19 dpi (A) and 35 dpi (H). At ca. 14 dpi, *nodB* was strongly expressed in the fixation zone (B) and in the infection zone (D), while *nifH* was expressed only in the fixation zone (C and E). At 35 dpi, bacteroids were randomly organized within the nodule cell (F) and symbiosomes contained up to 4 bacteroids (G) (arrows) with poly-β-hydroxybutyrate (PHB) granules (star). At 42 dpi, the infected zone was mainly restricted to the distal part of the nodule (H). At ca. 52 dpi, gene expression occurred only in disparate cells (I) and bacteria degenerated (J). Green, GFP; m, meristem; iz, infection zone; fz, fixation zone.

bacteria (Fig. 1C). As expected, *nifH* is expressed only in the fixation zone (Fig. 1C and E). Interestingly, *nodB* is expressed in rhizosphere (data not shown) and infection thread colonizing bacteria as well as in bacteroids, a rare situation in the rhizobium-legume symbiosis (Fig. 1B and D) (14). Contrary to the case with *Medicago* indeterminate nodules, symbiosomes in *M. pudica* did not exhibit radial organization (Fig. 1F) (16). They contained up to four bacteria harboring polyhydroxybutyrate polymers in their cytoplasm (Fig. 1G). After 42 dpi, a degenerating zone was observed, where gene expression occurred in disparate nodule cells (Fig. 1H and I). At ca. 52 dpi, only the degenerating zone persisted, where loss of cell-to-cell contact and cytoplasmic structure degradation of nodule cells could be observed (Fig. 1J).

We first evaluated morphological and DNA content changes undergone by *C. taiwanensis* strain 204 bacteroids compared to free-living bacteria grown in TY medium. Bacteroids were recovered at 35 dpi from nodules that had been previously surface sterilized, crushed in a phosphate-buffered saline (PBS) buffer, and centrifuged to eliminate most vegetal debris. Bacteria and

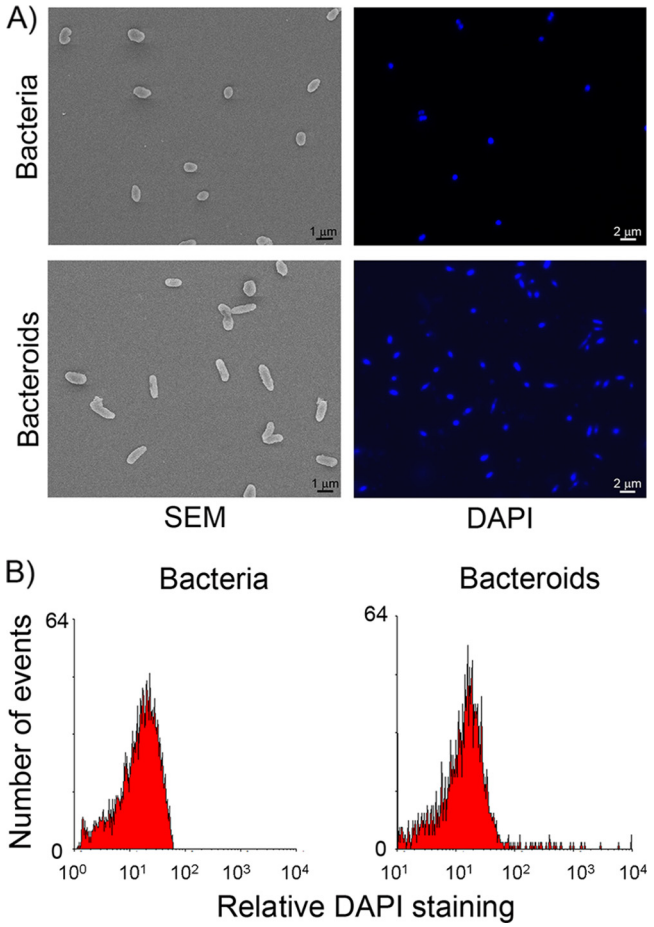

FIG. 2. Cell morphology and DNA content of *C. taiwanensis* free-living bacteria and bacteroids isolated from *M. pudica* nodules. (A) Scanning electron microscopy (SEM) of free-living bacteria and bacteroids isolated from 35-dpi nodules showed a 2-fold increase in bacterial size (left panel). Fluorescence microscopy of bacteria and bacteroids stained with DAPI is similar (right panel). (B) DNA content of DAPI-stained bacteria and bacteroids recovered from 35-dpi nodules as measured by flow cytometry showed no DNA amplification.

bacteroids were fixed in glutaraldehyde (4%) in cacodylate buffer, washed, dehydrated, and metallized (1.2 V, 10 mA). Scanning electron microscopy observation (MAB Hitachi S450 microscope) showed that bacteroids exhibited little morphological change (Fig. 2A), which was confirmed by Nomarski direct observation (data not shown). Bacteroids were indeed only slightly more elongated than free-living bacteria, being up to 2 times longer (1.7 to 2 μm, compared to 1 μm). Bacterial cells stained with the fluorescent DNA dye 4′,6-diamidino-2-phenylindole (DAPI) at 5 μg/ml were analyzed using fluorescence microscopy (Fig. 2A) and flow cytometry (Facscalibur) (Fig. 2B). No change in DNA content was observed, showing that *C. taiwanensis* bacteroids did not undergo genome amplification.

Bacteroid membrane integrity was evaluated using 2 μg/ml propidium iodide (PI), a DNA stain that enters cells with alteration of membrane permeability. No staining was observed for free-living bacteria or for bacteroids, while penetration of the dye was very rapid in bacteria killed at 95°C for 10 min (data not shown). Membrane permeability is thus not altered in *C. taiwanensis* bacteroids.

To evaluate the viability of intracellular bacteria, *M. pudica* plants were inoculated with *C. taiwanensis* (CBM2153) harboring a *nifH-gfp* fusion. *gfp*-positive bacteroids (5×10^3) were sorted by flow cytometry (FacsARIA II-SORP; BD) and subsequently counted using dilution series plated on selective TY medium supplemented with tetracycline at 10 μg/ml. Twenty percent of *gfp*-expressing cells were able to resume growth on TY medium.

Altogether these results showed that *C. taiwanensis* bacteroids are not terminally differentiated in *M. pudica* nodules, in sharp contrast with the profound and irreversible bacteroid differentiation observed in *Medicago* and other galegoid legumes of the Papilionoideae subfamily (9). We have here demonstrated that all of the characters associated with bacteroid differentiation in galegoid nodules, i.e., cell enlargement, polyploidy, membrane permeability modification, and loss of viability, are actually unrelated to nodule ontogeny.

In a recent study, Oono et al. (12), in a literature- and experimentally based overview of overall bacteroid morphology (swollen versus nonswollen) in the Papilionoideae subfamily, similarly concluded there was no correlation between bacteroid differentiation and nodule ontogeny.

One of the key genes for terminal bacteroid differentiation in galegoid nodules is *bacA*, which is involved in very long chain fatty acid (VLCFA) modification of the outer membrane and possibly peptide transport. It has been suggested that BacA may be involved, directly or indirectly, in the import of nodule-specific cysteine-rich (NCR) plant peptides (15) with antimicrobial activity, which indeed are extremely abundant in and specific to galegoid nodules (6a, 9). *C. taiwanensis* lacks any *bacA* gene, which is in agreement with the fact that this beta-rhizobium does not undergo terminal bacteroid differentiation in symbiosis with *Mimosa pudica*.

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