Cupriavidus taiwanensis bacteroids in Mimosa pudica indeterminate nodules are not terminally differentiated.

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Running title: Cupriavidus taiwanensis bacteroid differentiation

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SUMMARY

The β-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.

TEXT:

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 Cesalpinoideae subfamilies. Rhizobia are phylogenetically disparate bacteria distributed in many genera of α- and β-proteobacteria (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 from 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 from external cortical cells, 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 size enlargement (mean 5x), DNA amplification (mean 24C) and modification in membrane permeability and loose their capacity for reproduction (<1%)(9). By contrast, bacteroids of determinate nodules of *Phaseolus*, *Lotus* or soybean poorly differ from free-living bacteria (9). Both histological types of nodules and bacteroid differentiation level are controlled by the legume host (9, 11). It is however unclear so far whether nodule ontogeny and bacteroid differentiation are truly correlated.

We investigated this issue on an atypical model system. The β-rhizobium *Cupriavidus* (formerly *Ralstonia*) *taiwanensis* nodulates *Mimosa pudica* (1, 4) a plant of the *Mimosoideae* subfamily that forms indeterminate nodules (3). As most rhizobia, *C. taiwanensis* penetrates root tissues via root hairs from which infection threads elongate towards the emerging nodule. Bacteroids are released from infection threads in the cytoplasm of nodule cells where they are enclosed in a symbiotic structure called symbiosome. So far the extent of bacteroid differentiation in *M. pudica* nodules has not been investigated.
We confirmed that in our experimental conditions *M. pudica* formed indeterminate nodules. Seedlings of *M. pudica* were grown in Gibson tubes in N-free conditions and inoculated by $10^7$ bacterial cells grown in TY as previously described (7) except that Gibson tubes contained only quarter-strength liquid Jensen. Either one of the following *C. taiwanensis* LMG19424 derivatives was used as inoculum: strain 204, which constitutively expresses *gfp* (4), strain CBM132 which contains a plasmidic *nodB-lacZ* fusion (7), strain CBM722 which contains the pCBM39 plasmid harbouring a *nifH-lacZ* fusion and strain CBM2153 which contains the pCBM78 plasmid harbouring a *nifH-gfp* fusion. To construct pCBM39, the *nifH* promoter of *C. taiwanensis* was amplified using 5’-CCCAGCTTGGATGCTAGCGAAGCG-3’ and 5’-TGCACTGCAGCCATTTTGAATTGAAGGTGTAGC-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’-TGCACTGCAGCAGCAGCCAACCTGC-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 (7). Nodules emerged from the inner cortex (data not shown). At ca. 14 post-inoculation (dpi), nodules harboured 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, 1D) and a fixation zone where nodule cells were massively infected by bacteria (Fig. 1C). As expected, *nifH* is only expressed in the fixation zone (Fig. 1C, 1E). 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, 1D) (14). Contrarily to *Medicago* indeterminate nodules, symbiosomes in *M. pudica* did not exhibit radial organisation (Fig. 1F)(16). They contained up to four bacteria harbouring 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, 1I). 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 as compared to free-living bacteria grown in TY medium. Bacteroids were recovered at 35 dpi from nodules that have been previously surface sterilized, crushed in a PBS buffer and centrifuged to eliminate most of vegetal debris. Bacteria and bacteroids were fixed in glutaraldehyde 4% in cacodylate buffer, washed, dehydrated and metallised (1.2 volts, 10 mA). Scanning electron microscopy observation (MAB Hitachi S450 microscope) showed that bacteroids exhibited little morphological changes (Fig. 2A), which was confirmed by Normaski direct observation (data not shown). Bacteroids were indeed only slightly more elongated than free-living bacteria, being up to 2 times longer (1.7-2 µm compared to 1 µm). Bacterial cells stained with the fluorescent DNA dye 4’,6-diamidino-2-phenylindole (DAPI) at 5µg/ml were analysed 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 as well as 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) harbouring a nifH-gfp fusion. 5x10³ gfp-positive bacteroids were sorted by flow cytometry (Facs ARIA II –SURP BD) and subsequently counted using dilution series plated on selective TY medium supplemented with tetracycline 10µg/ml. 20% 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 permability 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 vs non-swollen) in the Papilionoideae sub-family similarly concluded to the absence of correlation between bacteroid differentiation and nodule ontogeny.

One of the key genes for terminal bacteroid differentiation in galegoid nodules is bacA that 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 that are indeed extremely abundant in and specific of galegoids nodules (9, 13). *C. taiwanensis* lacks any bacA, which is in agreement with the fact that this β-rhizobium does not undergo terminal bacteroid differentiation in symbiosis with *Mimosa pudica*.

We are grateful to Ton Timmers for careful reading of the manuscript. We thank Fatima-Ezzahra L’Faqihi-Olive and Valérie Duplan-Eche from the IFR150 cytometry platform and Bruno Payré and Isabelle Fourquaux from the IFR-BM CMEAB platform for helpful technical assistance. This work was supported by grants from SPE INRA department and ANR-08-BLAN-0295-01.

**Figure legends**

**Figure 1. Imaging of indeterminate nodules formed by C. taiwanensis.**

Fluorescence (I), light (B, C, D, E, H, J), and electron (F, G) microscopy of nodules formed by *C. taiwanensis* genomic GFP (A, I, F, G), plasmidic nodB-lacZ (B, D, H) and plasmidic nifH-lacZ (C, E) or stained with toluidine blue (A, 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 only expressed in the fixation zone (C, E). At 35 dpi, bacteroids were randomly organized within the nodule cell (F) and symbiosomes contained up to 4 bacteroids (E).
(arrows) with 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. Red, autofluorescence of plant cells. m, meristem. iz, infection zone. fz, fixation zone.

Figure 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 35dpi nodules as measured by flow cytometry showed no DNA amplification.

References


FIGURE 2