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'*Candidatus* Glomeribacter gigasporarum'
gen. nov., sp. nov., an endosymbiont of
arbuscular mycorrhizal fungiValeria Bianciotto,¹ Erica Lumini,¹ Paola Bonfante¹ and Peter Vandamme²¹Istituto per la Protezione delle Piante (Sezione di Torino) del CNR and Dipartimento di Biologia Vegetale dell'Università di Torino, Viale Mattioli 25, 10125 Torino, Italy²Laboratorium voor Microbiologie, Faculteit Wetenschappen, Universiteit Gent, Gent, Belgium

Arbuscular mycorrhizal fungi are obligate endosymbionts that colonize the roots of almost 80 % of land plants. The present paper describes morphological and molecular data on a bacterial endosymbiont living in the cytoplasm of dormant or germinating spores and symbiotic mycelia of the fungal species *Gigaspora margarita*, *Scutellospora persica* and *Scutellospora castanea*. PCR amplification of almost the entire 16S rRNA gene of the *Gigaspora margarita* BEG 34 endosymbiont, using universal bacterial primers, and subsequent sequence analysis demonstrated that this organism occupies a very distinct phylogenetic position within the β -Proteobacteria, with the genera *Burkholderia*, *Pandoraea* and *Ralstonia* as its closest neighbours. Primers specific to the 16S rDNA of the endosymbiotic bacteria of BEG 34 allowed amplification of spore DNA from endosymbionts of *Gigaspora margarita*, *Gigaspora decipiens*, *S. persica* and *S. castanea*, but not from the *Gigaspora gigantea* endosymbiont (which was morphologically different) or from the cytoplasm of *Gigaspora rosea* (which did not contain endosymbiotic bacteria). These specific primers were successfully used as a probe for the *in-situ* hybridization of endobacteria in *Gigaspora margarita* spores. The overall rod-shaped morphology of the *Gigaspora margarita*, *Gigaspora decipiens*, *S. persica* and *S. castanea* endosymbionts was similar, and amplification and sequence analysis of the almost-complete 16S rRNA genes of several *Gigaspora margarita*, *S. persica* and *S. castanea* endosymbionts revealed over 98 % sequence similarity. These morphological and genomic characteristics were used to assign the endosymbionts of these three species (five isolates) of arbuscular mycorrhizal fungi as '*Candidatus* Glomeribacter gigasporarum' gen. nov., sp. nov.

Arbuscular mycorrhizal fungi live in obligate symbiosis with the roots of about 80 % of land plants. Present in most natural and agricultural ecosystems, they are important for plant health, nutrient cycling and conservation of soil structure (Read *et al.*, 1992). They belong to a new fungal phylum, the Glomeromycota (Schüßler *et al.*, 2001), which may have been instrumental in the colonization of the land by ancient plants (Pirozynski & Malloch, 1975). The cytoplasm of arbuscular mycorrhizal fungi harbours structures called bacteria-like organisms (Mosse, 1970), which have been observed by electron microscopy in different arbuscular mycorrhizal fungal species (*Glomus versiforme*, *Acaulospora laevis*, *Gigaspora margarita*) (Bonfante *et al.*, 1994; MacDonald & Chandler, 1981; Mosse *et al.*, 1970; Scannerini & Bonfante, 1991). In spite of numerous attempts, these organisms have never been grown on

cell-free media (MacDonald & Chandler, 1981; Scannerini & Bonfante, 1991). However, the morphological characteristics and several genomic characteristics of some of these organisms, which were shown to be bacterial endosymbionts, have been described in recent studies (Bianciotto *et al.*, 1996, 2000; Minerdi *et al.*, 2001, 2002a, b; Ruiz-Lozano & Bonfante, 1999, 2000). The present paper summarizes the taxonomic findings of an endosymbiont present in several *Gigaspora* and *Scutellospora* species, in order to assign this bacterium formally to the provisional *Candidatus* designation for uncultured bacteria (Murray & Schleifer, 1994; Murray & Stackebrandt, 1995).

***Gigaspora margarita* BEG 34 endosymbiont**

In 1996, Bianciotto *et al.* (1996) reported the morphological and genotypic characteristics of rod-shaped prokaryotic cells that occurred in spores of *Gigaspora margarita* isolate BEG 34. The spores were recovered from pot cultures of *Trifolium repens* L. (clover), rinsed and surface-sterilized.

The GenBank accession numbers for the 16S rRNA gene sequences of the '*Candidatus* Glomeribacter gigasporarum' strains are X89727 and AJ251633–AJ251636.

Fig. 1. Transmission electron micrographs of bacteria (B) contained in vacuoles (V) in the cytoplasm of the arbuscular mycorrhizal fungus *Gigaspora margarita* BEG 34. (a) Transversally sectioned bacteria: note the prokaryotic layered cell wall. Bar, 0.2 μ m. (b) Longitudinal and transverse sections of endobacteria, showing a cytoplasm rich in ribosomes (arrows). Bar, 0.15 μ m.

Sterilized clover seeds had been sown in sterilized quartz sand, and mycorrhizal plants were obtained by injecting a sterilized suspension of *Gigaspora margarita* spores around the seedlings.

Spores of *Gigaspora margarita* and mycorrhizal roots of clover prepared after high-pressure/freeze-fixation and examined by using electron microscopy (Bonfante *et al.*, 1994) revealed, in the vacuoles, large numbers of rod-shaped bacteria-like organisms with a laminated cell wall and a cytoplasm rich in ribosomes (Fig. 1a, b). Organisms with similar morphology, including cells undergoing division, were observed in vacuoles within germinating mycelium and within the intraradical hyphae produced during root infection. Analysis of unfixed crushed spores after staining with the Bacteria Counting kit (Molecular Probes) confirmed that the bacteria-like organisms were indeed rod-shaped bacteria which fluoresced as green (Fig. 2). At higher magnification, many bacteria appeared in division (inset, Fig. 2).

Amplification of bacterial 16S RNA genes with universal prokaryotic primers 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTACGA-3') (Bianciotto *et al.*, 1996) from total spore DNA of *Gigaspora margarita* BEG 34, followed by direct sequence analysis, revealed a homogeneous bacterial population closely related to the genus *Burkholderia*, a member of the β -Proteobacteria. This 16S rDNA sequence (GenBank accession no. X89727) was used to design primers BLOf [5'-CACAGGTT(TG)AAACACTGGGT-3'] and BLOr (5'-GTCATCCACTCCGATTATTTA-3') for the specific amplification of a 411 bp fragment of the endosymbiotic DNA. PCR analysis gave products of the expected length from spores, external mycelium and from clover roots mycorrhized with *Gigaspora margarita* BEG 34, but not from any of the negative controls (DNA extracted from *Gigaspora rosea* spores and washing solutions of the spores).

These observations demonstrated that *Gigaspora margarita* BEG 34 harboured a large homogeneous bacterial population that was present in all stages of the fungal life-cycle. These

bacteria were an integral part of the fungal system, where they formed a large surface area that could serve for metabolic exchange.

Distribution of bacterial endosymbionts in other arbuscular mycorrhizal fungi belonging to the Gigasporaceae

In order to understand whether intracellular bacteria occurred sporadically in individual arbuscular mycorrhizal fungal isolates or were a common feature of the family Gigasporaceae, Bianciotto *et al.* (2000) subsequently investigated two additional isolates of *Gigaspora margarita* (derived from distinct geographical areas), four isolates of *Gigaspora rosea*, one isolate each of *Gigaspora gigantea* and *Gigaspora decipiens*, two isolates of *Scutellospora persica* and one isolate of *Scutellospora castanea*.

Fig. 2. Numerous bacterial endosymbionts separated from the fungal cytoplasm by crushing and centrifugation of *Gigaspora margarita* BEG 34 spores. The endobacteria-enriched solution was stained with the Bacteria Counting kit (Molecular Probes) and observed under an Olympus Fluoview confocal microscope. Bacteria fluoresce bright green under blue light. Bar, 5 μ m. Inset: bacterial cells in division; bar, 2 μ m.

Intracellular bacteria were observed in all fungal isolates except for the four *Gigaspora rosea* isolates. The overall morphology of the endosymbiotic cells was similar, except for those in *Gigaspora gigantea*, which were rounder and smaller. *In-situ* hybridization experiments using the oligonucleotide rDNA sequence BLOr were performed on three fungal isolates [two of *Gigaspora margarita* (BEG 34 and WV 205A) and one of *Gigaspora rosea* (BEG 9)] and confirmed, in *Gigaspora margarita* isolates, the identity and location of endobacteria in arbuscular mycorrhizal spores. A positive signal was obtained in *Gigaspora margarita* BEG 34 and *Gigaspora margarita* WV 205A, but not in the cytoplasm of *Gigaspora rosea*. Amplification of endobacterial 16S rDNA with universal prokaryotic primers 27f and 1495r (Bianciotto *et al.*, 1996) generated fragments for all fungal isolates in which endobacteria were observed. Application of the BLOf/BLOr primer pair in a PCR test generated fragments for the same isolates, except for the *Gigaspora gigantea* endosymbiont, which was also morphologically atypical.

The endosymbionts of four fungal isolates were examined further. Amplification and sequence analysis of almost the complete 16S rDNA of the *S. persica* HC/F E28, *S. persica* HC/F E09, *S. castanea* BEG 1 and *Gigaspora margarita* WV 205A endosymbionts (GenBank accession numbers AJ251634, AJ251635, AJ251636 and AJ251633, respectively) and of the *Gigaspora margarita* BEG 34 endosymbiont revealed over 98 % sequence similarity (Fig. 3). These five sequences formed a very distinct lineage within the β -Proteobacteria, with the genera *Burkholderia*, *Pandoraea* and *Ralstonia* as the closest neighbours. A 100 % bootstrap

value confirmed that the phylogenetic position of these endosymbionts was stable and that the arbuscular mycorrhizal fungal endosymbionts were different from the unculturable endosymbionts from leaf galls of *Psychotria* species (plant species belonging to the angiosperm family Rubiaceae), which are genuine members of the genus *Burkholderia* ('*Candidatus B. kirkii*' in Fig. 3; Van Oevelen *et al.*, 2002).

Other genomic characteristics

The finding that a genomic library developed from *Gigaspora margarita* spores was also representative of the endosymbiont genome (van Buuren *et al.*, 1999) helped to identify some of its genomic features. Among the bacterial genes identified so far, the most interesting are those involved in nutrient uptake (i.e. a putative phosphate transporter operon, *pst*) and a gene (*vac*) involved in colonization events by bacterial cells (Ruiz-Lozano & Bonfante 1999, 2000). A DNA region containing putative *nif* genes was also found (Minerdi *et al.*, 2001). Further analysis revealed three open reading frames encoding putative proteins with a very high degree of sequence similarity to the two subunits (NifD and NifK) of component I, and to component II (NifH) of nitrogenase from different diazotrophs. The three genes were arranged in an operon similar to that shown by most archaeal and bacterial diazotrophs.

In addition, the random sequencing method was used to obtain additional genomic information and revealed the presence of open reading frames encoding 'orphan' proteins (having no sequence similarity to proteins available in databases) and putative proteins showing similarity to other bacterial proteins. These proteins were involved in chemotaxis (CheY), the signal transduction pathway (McpA), kinases (PrkA), host-specificity proteins (bacteriophage λ protein J), sporulation proteins (SpoVR) and proteins with unknown functions (Minerdi *et al.*, 2002a, b). While the first two hypothetical proteins raise some interesting questions as to the capacity of these endosymbionts to sense the nutrient source, the others suggest the presence of proteins involved in basal cell metabolism and taxis. Interestingly, the proteins with no sequence similarity to database proteins did not show any homology with the open reading frames identified in the *Buchnera* genome, which, together with the *Rickettsia* genome, represent the only fully sequenced genomes of uncultivable microbes (Shigenobu *et al.*, 2000). However, it is important to underline that direct evidence is not yet available that all these genes belong to the genome of the novel *Candidatus* taxon.

'Candidatus Glomeribacter gigasporarum' gen. nov., sp. nov.

According to Murray & Schleifer (1994) and Murray & Stackebrandt (1995), the properties of uncultured organisms should be recorded under a *Candidatus* designation. Our data demonstrate that the endosymbionts of *Gigaspora margarita*, *S. persica* and *S. castanea* represent a single

Fig. 3. Neighbour-joining unrooted tree showing the phylogenetic position of the endobacteria belonging to the novel *Candidatus* taxon (boxed). The names included in the box are those of the fungal host strains carrying the '*Candidatus Glomeribacter gigasporarum*' endosymbionts. The tree was obtained from 16S rDNA sequences of the endosymbionts of *Gigaspora margarita*, *S. castanea* and *S. persica* isolates and the closest bacterial sequences retrieved by a BLAST search. Sequences were aligned by using CLUSTAL X, and the alignment was edited with GeneDoc. Neighbour-joining analysis was performed with CLUSTAL X using Kimura's distance method.

taxon with a similar morphology. This endosymbiont can be detected and identified using the BLOf/BLOr primer pair in a PCR test. The digoxigenin-labelled BLOr oligonucleotide can be used for *in-situ* hybridization experiments. The present data also suggest that the *Gigaspora gigantea* endosymbiont represents a distinct *Candidatus* taxon, for which no formal name is proposed pending the availability of almost the complete 16S rDNA sequence and a specific probe.

The endosymbiont of *Gigaspora margarita*, *S. persica* and *S. castanea* can be described as follows: '*Candidatus* Glomeribacter gigasporarum' gen. nov., sp. nov. (Glo.me. ri.bac'ter. L. gen. n. *glomeris* of a cluster; N.L. masc. n. *bacter* bacterium; N.L. n. *Glomeribacter* clustered bacterium; gi.ga.spo.ra'rum. N.L. gen. pl. n. *gigasporarum* of species of *Gigaspora*, a fungal genus) [(β -Proteobacteria) NC; G-; R; NAS (GenBank nos X89727, AJ251634, AJ251635, AJ251636 and AJ251633); oligonucleotide sequence complementary to unique region of 16S rRNA gene 5'-GTCATCCA-CTCCGATTATTTA-3', S (*Gigaspora margarita*, *S. persica* and *S. castanea*, fungal species belonging to the order Diversisporales, cytoplasm of spores and mycelium)] (Bianciotto *et al.*, 1996, 2000).

Cells are Gram-negative, rod-shaped and $0.8\text{--}1.2 \times 1.5\text{--}2.0\text{ }\mu\text{m}$ in size [dimensions were measured under fluorescence using the Counting Bacteria kit (Molecular probes) and a Olympus FluoView confocal microscope]. They occur singly or in groups surrounded by vacuole membranes.

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