An Obligately Endosymbiotic Mycorrhizal Fungus Itself Harbors Obligately Intracellular Bacteria

VALERIA BIANCIOTTO,[1] CLAUDIO BANDI,[2,3] DANIELA MINERDI,[1] MASSIMO SIRONI,[2]
HANS VOLKER TICHY,[4] AND PAOLA BONFANTE[1]*

*Dipartimento di Biologia Vegetale dell'Università di Torino and Centro di Studio sulla Micologia del Terreno
del Consiglio Nazionale delle Ricerche, 10125 Turin,[1] Istituto di Patologia Generale Veterinaria
dell'Università di Milano, 20133 Milan,[2] and Dipartimento di Genetica e Microbiologia
dell'Università di Pavia, 27100 Pavia,[3] Italy, and Fachgruppe Biologische Sicherheit,
TUV Energie und Umwelt GmbH, 79108 Freiburg, Germany[4]*

Arbuscular-mycorrhizal fungi are obligate endosymbionts that colonize the roots of almost 80% of land plants. This paper describes the employment of a combined morphological and molecular approach to demonstrate that the cytoplasm of the arbuscular-mycorrhizal fungus *Gigaspora margarita* harbors a further bacterial endosymbiont. Intracytoplasmic bacterium-like organisms (BLOs) were detected ultrastructurally in its spores and germinating and symbiotic mycelia. Morphological observations with a fluorescent stain revealed about 250,000 live bacteria inside each spore. The sequence for the small-subunit rRNA gene obtained for the BLOs from the spores was compared with those for representatives of the eubacterial lineages. Molecular phylogenetic analysis unambiguously showed that the endosymbiont of *G. margarita* was an rRNA group II pseudomonad (genus *Burkholderia*). PCR assays with specifically designed oligonucleotides were used to check that the sequence came from the BLOs. Successful amplification was obtained when templates from both the spores and the symbiotic mycelia were used. A band of the expected length was also obtained from spores of a *Scutellospora* sp. No bands were given by the negative controls. These findings indicate that mycorrhizal systems can include plant, fungal, and bacterial cells.

Arbuscular-mycorrhizal (AM) 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 (29). They belong to an order within the Zygomycetes, the Glomales, whose origin and divergence among the main genera have been dated between 400 and 100 million years ago (32), and they may have been instrumental in colonization of the land by ancient plants (28). The close relationship of AM fungi with their host plants is mirrored by their obligatory biotrophic status. In the absence of the host, their growth is limited, while inside the root cells, they display a complex host-regulated morphogenesis (5).

The cytoplasm of AM fungi harbors structures called bacterium-like organisms (BLOs) (24), whose identification has been hampered because they cannot be grown on cell-free media (20, 30). This study investigates their morphology, distribution, and phylogenetic position through a combination of morphological analyses (transmission electron microscopy and confocal microscopy) and the molecular analysis of the gene coding for the small-subunit rRNA, 16S rDNA. The latter approach has been widely used to investigate the phylogeny of uncultured symbiotic organisms (1).

MATERIALS AND METHODS

Biological materials: fungi, mycorrhizal roots, and bacteria. Spores of *Gigaspora margarita* were recovered from pot cultures of *Trifolium repens* L. (clover) by wet sieving (15), rinsed five times with sterile, filtered and distilled water, surface

sterilized with 4% of Chloramine-T and 300 ppm of streptomycin for 30 min, and then rinsed five times for 1 h with sterile, filtered and distilled water.

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

All of the other spores were obtained in the same way from plants infected with fungal isolates of different origins (Table 1). A specimen voucher for each sample was deposited in the Herbarium Cryptogamicum of the Dipartimento di Biologia Vegetale dell'Università di Torino. Spores of *Geosiphon pyriforme* were kindly provided by Arthur Schussler (University of Heidelberg), while fruiting bodies from *Tuber borchii* Vitt., an ectomycorrhizal fungus, were collected in the field. Four species of soil bacteria were included as controls, namely, *Agrobacterium tumefaciens*, *Rhizobium meliloti* GR4, *Enterobacter agglomerans*, and *Pseudomonas fluorescens* CHA0. The bacteria were provided by B. Lugtenberg, N. J. Brewin, and G. Défago.

Morphological observations. Spores of *Gigaspora margarita* and mycorrhizal roots of clover were prepared after high-pressure freezing and freeze substitution. The samples were vacuum infiltrated in 20 mM CaCl₂–2 mM KCl–0.2 M sucrose and then frozen at high pressure with a Balzers HPM 010 apparatus. Substituted samples prepared as described by Bonfante et al. (4) were examined with a Philips CM10 transmission electron microscope at 80 kV.

A cryomicrotome (Micron-Heidelberg) was used to provide 20-μm-thick sections of unfixed spores of *Gigaspora margarita* for staining with a Live/Dead *Bac*Light Bacteria Viability Kit (Molecular Probes Europe BV) as described in the manufacturer's instructions by incubation at room temperature in the dark for 15 min, followed by immediate examination with a ViewScan DVC-250 (Bio-Rad Microsciences Ltd.). This kit contains a proprietary mixture of nucleic acid stains that distinguishes live bacteria with intact plasma membranes, which fluoresce green, from dead bacteria with damaged membranes, which fluoresce red, and thus allowed their detection without fixing or washing steps. Counting of the live BLOs was done on optical sections (median thickness, 0.6 μm) a from five spores produced by a confocal microscope equipped with numerical aperture 1.4 lenses.

PCR conditions and sequencing. For crude DNA preparation, samples of 50 spores were rigorously surface decontaminated (see above), thoroughly washed in filtered sterilized water, resuspended in an 80-μl volume of 10 mM Tris-HCl (pH 7.5), overlaid with mineral oil, heated at 90°C for 10 min, crushed with a plastic pestle, treated with proteinase K (final concentration, 100 μg/ml in a 100-ml volume) at 65°C for 15 min and then at 50°C for 3 h, and heated again at 90°C for 10 min. They were then centrifuged at 12,000 × *g* for 5 min, and the supernatant was recovered. Extreme care was taken to avoid bacterial contamination. All solutions were filter sterilized, and sterile procedures were used

* Corresponding author. Mailing address: Dipartimento di Biologia Vegetale dell'Università and Centro di Studio sulla Micologia del Terreno del C.N.R., Viale Mattioli 25, 10125 Torino, Italy. Phone: 39 11 6502927. Fax: 39 11 655839. Electronic mail address: Bonfante@CSMT.TO.CNR.IT.

TABLE 1. Isolates of AM fungi

Isolate	Origin	Obtained from[a]:	Voucher[b]
Glomus mosseae (Nicol. & Gerd.) Gerd. & Trappe	Rothamstead, United Kingdom	A. Schubert (Turin, Italy)	HC/F-E05
Scutellospora sp. (Schenck & Nicol.) Walker & Sanders	Migliarino, Italy	M. Giovannetti (Pisa, Italy)	HC/F-E11
Gigaspora margarita Becker & Hall	New Zealand (J. R. Crush)	V. Gianinazzi-Pearson (Dijon, France) (LPA 2)	HC/F-E10
Acaulospora laevis Gerd. & Trappe	Rothamstead, United Kingdom	V. Gianinazzi-Pearson (Dijon, France) (LPA 1)	HC/F-E09

[a] For some of the fungal isolates, the isolate numbers from the collections of origin are provided in parentheses.
[b] Vouchers have been deposited in the Herbarium Cryptogamicum (HC) of the Dipartimento di Biologia Vegetale dell'Università di Torino.

during crushing. Bacterial 16S rDNA was amplified in a 20-μl volume containing 2 μl of crude DNA preparation, 1 μM eubacterial primers 27f (5'-GAGAGTT TGATCCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTTACGA-3'), 2 μl of 10$\times$ buffer (Dynazyme; Celbio), 200 μM each deoxynucleoside triphosphate, and 1 U of Super *Taq* Polymerase (Stehelin). PCR cycling conditions were 94°C for 30 s, annealing temperature for 30 s, and 72°C for 4 min; the annealing temperature was 60°C for the first five cycles, 55°C for the next five cycles, and 50°C for last 25 cycles (2). Direct sequencing of PCR products was done with eubacterial primers 27f, 704f (5'-GTAGCGGTGAAATGCGTAGA-3'), 765r (5'-CTGTTTGCTCCCCACGCTTTC-3'), 1495r, 519r [5'-G(AT)ATTACCGC GGCGGC(GT)GCTG-3'], and 926f [5'-AAACT(CT)AAA(GT)GAATTGACG G-3'] (2, 18) with a Circum Vent DNA sequencing kit (New England Biolabs), as described in the manufacturer's recommendations.

Two primers, BLOf [5'-CACAGGTT(TG)AAACACTGGGT-3'] and BLOr (5'-GTCATCCACTCCGATTATTTA-3'), were designed with the sequenced fragment and expected to amplify a 411-bp fragment. These primers were specifically designed for the BLOs of *Gigaspora margarita* after comparison with the available small-subunit (SSU) entries of the Ribosomal Database Project (21). Cross-hybridizations with these other 16S rDNA sequences at the 3' end of the primers are unlikely.

PCR amplifications with 704f and 1495r and with BLOf and BLOr were performed on DNA preparations from AM (Table 1) and *Geosiphon pyriforme* spores. The same primers were used on the following controls: ectomycorrhizal fruiting bodies, soil bacteria, and sand from the pot cultures. Primers 704f and 1495r were used to check for the presence and accessibility of eubacterial DNA. Primers BLOf-BLOr and 704f-1495r were also employed with DNA preparations from the external mycelium of *Gigaspora margarita* and from clover roots infected with *Gigaspora margarita*. Negative controls were also set up by excluding the template. PCR conditions were as described above (2). Primers were obtained from Primm s.r.l. (Milan, Italy). DNA fragments amplified from spores, external mycelium, and mycorrhizal roots with BLO-specific primers were digested with two restriction enzymes (*Hin*fI and *Sau*3AI) at 37°C for 4 h.

After a scan for its similarity with sequences in the EMBL data bank, the sequence was checked for secondary structural constraints and manually aligned with groups of prealigned 16S rDNA sequences (21) representing the main eubacterial lineages (38). Phylogenetic analysis was conducted by use of distance matrix (neighbor-joining and Fitch-Margoliash methods; Jukes & Cantor and Kimura corrections) and character state (maximum parsimony and maximum likelihood) (see references cited by Swofford and Olsen [34]) procedures by using the TREECON 3.0 (36) and PHYLIP 3.5c (13) programs on sequence alignments including or excluding the 16S rDNA variable regions (see appendix 1 in reference 18) and/or the insertions and deletions. Percentages of nucleotide divergences were estimated by the model of Kimura by taking the insertions and deletions into account, as outlined by Van de Peer et al. (37).

Nucleotide sequence accession number. The 16S rDNA sequence for the *Gigaspora margarita* endosymbiont has been deposited in the EMBL data library (accession number X89727).

RESULTS

The morphology of germinating spores of *Gigaspora margarita* is demonstrated in Fig. 1a and b. Transmission electron microscopy revealed large numbers of rod-shaped BLOs in the spore vacuoles (Fig. 1c and d). They possessed a laminated wall and cytoplasm rich in ribosomes. BLOs with a similar morphology were observed within germinating mycelium and the intraradical hyphae produced during root infection (Fig. 1e), once again in vacuoles (Fig. 1f and g). They were also observed undergoing division (data not shown).

The fluorescence kit specific for bacteria detected the BLOs on frozen spore sections and confirmed their bacterial nature (Fig. 2). They mostly fluoresced as yellow-green, rod-shaped spots (Fig. 2a and c). There were only a few dead (fluoresced red) cells (Fig. 2b).

We counted the number of live bacteria on optical sections produced by a fluorescence confocal microscope. We used median optical sections from five spores; the total volume analyzed was about $75 \cdot 10^3$ μm^3. The mean number of bacteria was 30.78 ± 0.66 per 10 μm^3. This gave a total of about 250,000 live bacteria for the approximately $8 \cdot 10^6$-μm^3 cytoplasm of the whole spore.

To investigate their taxonomic position, eubacterial primers were used for PCR amplification of almost the whole 16S rDNA. The amplified products were directly sequenced to overcome ambiguities due to the incorporation of errors in *Taq* polymerase synthesis. A 500-bp portion of the sequence (at the 5' end) was obtained independently in two laboratories (Pavia, Italy, and Freiburg, Germany) from different batches of *Gigaspora margarita* spores. Identical sequences were obtained, indicating that the populations contained a single bacterial type. Nearly the entire 16S rDNA sequence (1,423 bp) was obtained from one batch.

Two primers (BLOf and BLOr) were designed to be specific for the sequence. To monitor the fate of the BLOs during the fungal life cycle, these primers were used in PCR assays on resting spores, external mycelium, and clover roots infected with *Gigaspora margarita*. To assess their specificity, controls were tested with both the eubacterial and the specific primer sets. These controls included nonmycorrhizal clover roots, a fungus (*Tuber borchii*) which harbors intercellular bacteria between hyphae within the fruiting body, four rhizosphere bacteria belonging to the genera *Pseudomonas*, *Rhizobium*, *Enterobacter*, and *Agrobacterium*, and sand from pot cultures (data not shown). The BLO primers gave products of the expected length (411 bp) from spores, external mycelium, and clover roots infected with *Gigaspora margarita*. No amplification was obtained from any of the controls (Fig. 3a and b). Furthermore, after digestion with the two restriction enzymes, 411-bp bands showed the same patterns (data not shown). Products of the expected length were obtained from the controls when the eubacterial primers were used, excluding the buffer used for washing the spores of *Gigaspora margarita* before crushing them, which gave no amplification (Fig. 3a and b). In another experiment, DNA from spores of other members of the order Glomales (*Acaulospora laevis*, *Glomus mosseae*, and a *Scutellospora* sp.) (Fig. 3c) and from *Geosiphon pyriforme* (data not shown) was amplified with the specific primers. A band of about 400 bp was obtained only from a *Scutellospora* sp. (Fig. 3c).

It should be noticed that the eubacterial primers used to check for the presence and accessibility of eubacterial DNA in the negative controls (704f and 1495r) were designed to am-
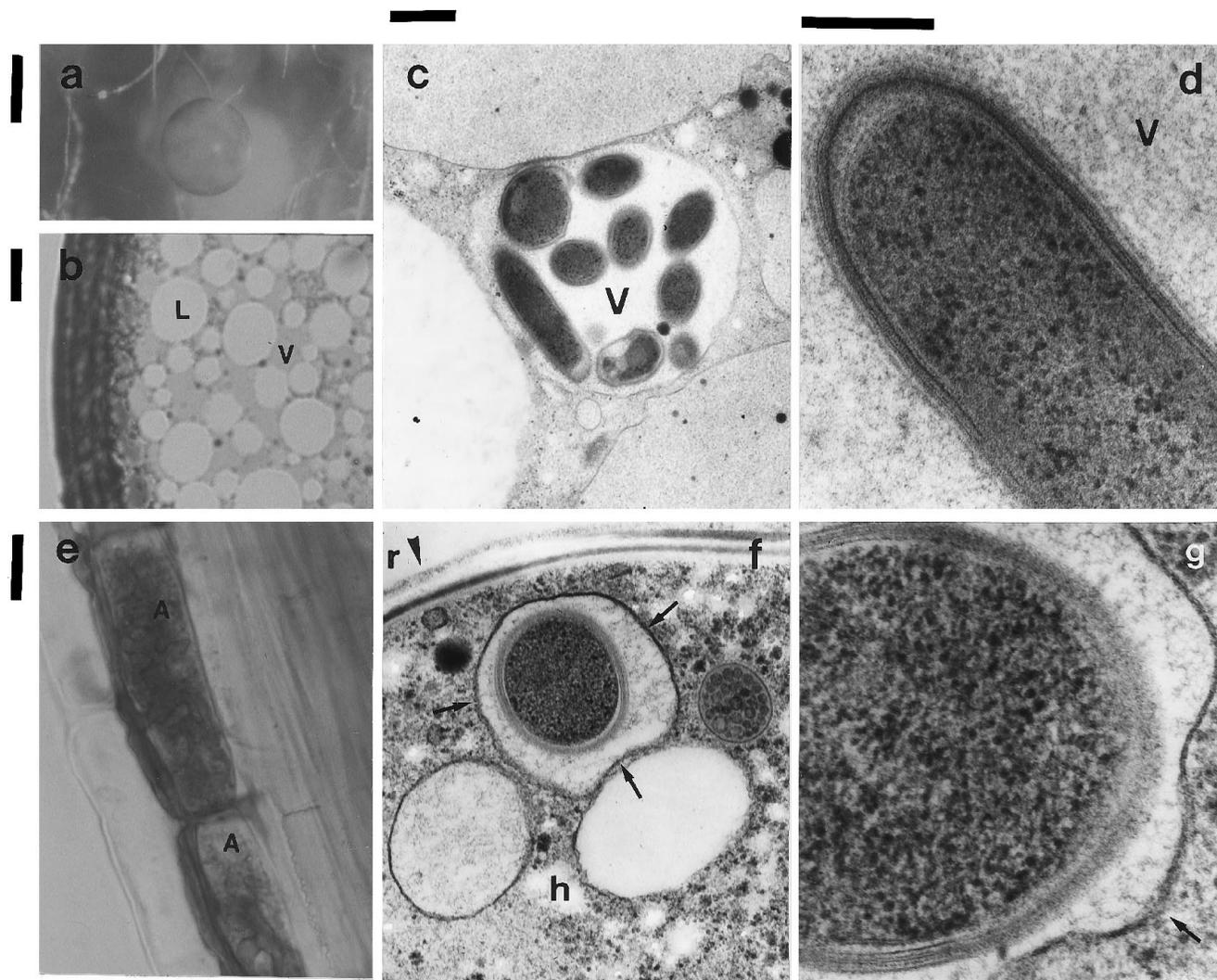
FIG. 1. Morphology of the endosymbiotic mycorrhizal fungus *Gigaspora margarita* and its endosymbiotic bacteria. (a) Fungal spore with germinating mycelium as seen under a stereomicroscope. Bar, 200 μm. (b) Lipid globules (L) and vacuoles (V) within which the bacteria occur. The section is stained with 1% toluidine blue. Bar, 15 μm. (c and d) Transmission electron micrographs of rod-shaped bacteria with a layered cell wall in the cytoplasm of *Gigaspora margarita* spores. V, vacuole. Bars, 0.5 (c) and 0.2 (d) μm. (e) *Gigaspora margarita* during colonization of clover roots as seen under a light microscope after staining with 1% cotton blue. The symbiotic hyphae produce highly branched structures called arbuscules (A). Bar, 20 μm. (f) Bacteria within a symbiotic hypha (h) inside a cortical root cell of the host (r) and separated from the plant cytoplasm by a membrane of plant origin (arrows). Bar, 0.2 μm. (g) Magnification of an intracellular bacterium separated from the fungal cytoplasm by the fungal vacuolar membrane (arrow). Bar, 0.2 μm.

plify a portion of the 16S rDNA from eubacterial but not from mitochondrial and chloroplast DNA of eukaryotic cells. Because it is impossible to clean roots of all soil bacteria, amplification from the uncolonized roots with the universal eubacterial primers is likely due to the presence of a few remaining bacterial cells.

Database searches for sequence similarity, sequence signature comparisons (38), and phylogenetic analyses of the 16S rDNA unambiguously placed the symbionts of *Gigaspora margarita* among the pseudomonads of rRNA group II (27), a eubacterial taxon belonging to the β subclass of the proteobacteria (19, 33). The tree in Fig. 4 (neighbor-joining method; Kimura's correction) is based on an alignment which included the 16S rDNA variable regions and took the insertions and deletions into account. Trees with the same placement for *Gigaspora margarita* endosymbionts were obtained after com-

plete exclusion or inclusion of both insertions and deletions and/or variable regions as well as by other methods (maximum parsimony and maximum likelihood) for tree construction. The average nucleotide divergence between the endosymbionts and the members of the *Burkholderia cepacia* branch (10% nucleotide substitutions) is consistent with an ancient separation of the lineage leading to the endosymbiont from that leading to free-living burkholderiae. Molecular rates of evolution calibrated for the bacterial 16S rDNA (1 to 2% every 50 million years) (23, 26) indicate that the split of the two lineages occurred approximately 125 to 250 million years ago.

DISCUSSION

This study demonstrates that *Gigaspora margarita* harbors a very large homogeneous population whose morphology and

FIG. 2. Bacterial endosymbionts in frozen sections of unfixed spores of *Gigaspora margarita* stained with the Live/Dead *Bac*Light Bacteria Viability Kit (Molecular Probes Europe BV). Living bacteria fluoresce bright yellow-green under blue light, while dead bacteria fluoresce red under green light. L, lipids. (a) Cytoplasm of a *Gigaspora margarita* spore exhibiting many living bacteria that fluoresce green lipids (arrows) and fungal nuclei (diffuse red masses). The wall is thick, laminated, and autofluoresces yellow. No contaminating bacteria were observed on the external surface of the sterilized spores. Bar, 25 μm. (b and c) At a higher magnification, bacterial endosymbionts are rod shaped (arrows). Under green light, dead bacteria fluoresce red (b); under blue light, many living bacteria fluoresce bright green (c). Bars, 10 (b) and 7 (c) μm.

genes coding for the small-subunit rRNA are indicative of true bacteria that are viable, multiply, and move from the spore via the mycelium towards the root of the host plant. PCR experiments showed that they are found in all stages of the fungal life cycle. They are an integral part of the fungal system, where they form a large surface area that could serve for metabolic exchanges. Moreover, this large number of BLOs contributes to increase the DNA content of AM fungi. The presence of prokaryotic sequences in genomic DNA extracted from AM spores should be considered, for example, during the construction and screening of DNA libraries.

Eukaryotic cells harboring high numbers of bacterial endosymbionts have been described for bivalves, insects, and protozoa, and the symbiotic bacteria have been identified for several of these organisms (12). However, few data are available for uncultured intracellular symbionts in fungi (8), and no phylogenetic studies have been reported. To our knowledge, *Geosiphon pyriforme* is the only example of symbiosis where a fungus harbors an intracellular prokaryote, namely, a nitrogen-fixing *Nostoc* sp. growing in pure culture (31). Interestingly enough, the symbiotic fungus displays a close relationship to AM fungi through its cell wall structure (31) and analysis of genes encoding for the small-subunit rRNA (14).

Many recent papers have described nitrogen-fixing bacteria in AM fungi and suggested that *Azospirillum* and *Acetobacter* species may play important roles in fertilization (17, 35). However, in our opinion, these investigations do not demonstrate the presence of intracellular bacteria. Bacteria could be present over the thickened and layered cell wall of AM spores (7). In truffles, too, bacteria living among (but not inside) the hyphae of the fruiting body have been identified (9).

By contrast, uncultured BLOs have been observed in many AM fungi (30) as well as in *Geosiphon pyriforme* (31) and in another zygomycete, *Endogone flammicorona* (6), but they have never been identified. Our phylogenetic 16S rDNA analysis unambiguously identifies the endosymbiont of *Gigaspora margarita* as a member of the genus *Burkholderia*, namely, a

pseudomonad of the rRNA group II (27). This eubacterial taxon belongs to the β subclass of the proteobacteria (19, 33) and includes plant and animal pathogens, rhizosphere bacteria, and nitrogen-fixing species (16, 27, 39). It consists of three branches, namely, the *B. cepacia*, *Burkholderia solanacearum*, and *Alcaligenes eutrophus* rRNA branches (16, 26) (Fig. 4). The name *Burkholderia* has recently been restricted to the *B. cepacia* branch (16). Comparison with the 16S rDNA sequences of these branches showed that the *Gigaspora margarita* BLOs are a sister group of the *B. cepacia* branch. This relationship was strongly supported by bootstrap analysis (94% after 100 replicates in the analysis shown in Fig. 4). Moreover, nucleotide divergence between the endosymbionts and group II pseudomonads was 10 to 13%, whereas the nearest other members of the β proteobacteria showed at least 17% divergence. The next step will be to see whether all the BLOs inside AM fungi are true bacteria.

rRNA genes are widely used to identify uncultured bacteria (1) and compare the phylogenies of hosts and symbionts (2, 3, 10, 11, 25). The specific primers designed for the *Gigaspora margarita* endosymbionts did not lead to amplification of bacterial DNA from the AM fungi *Glomus mosseae* and *Acaulospora laevis*, which are only distantly related to *Gigaspora margarita*. In addition, PCR experiments on *Geosiphon pyriforme*, which is regarded as a distant relative of AM fungi (14), were unsuccessful. By contrast, we observed amplification when using DNA templates from a *Scutellospora* sp., a member of the family Gigasporaceae, as well as *Gigaspora margarita*. The lack of amplification from other AM fungi could be due to mismatches at the 3′ end of the primers BLOf and BLOr (designed for the BLOs of *Gigaspora margarita*) but also to the presence of endosymbionts not related to the group II pseudomonads. In any case, bacterial endosymbionts could provide a new character source for studying the phylogeny of AM fungi.

The establishment of symbiosis, in its many forms, between prokaryotes and eukaryotes has been considered an important source of evolutionary innovation (22). Even if the functional
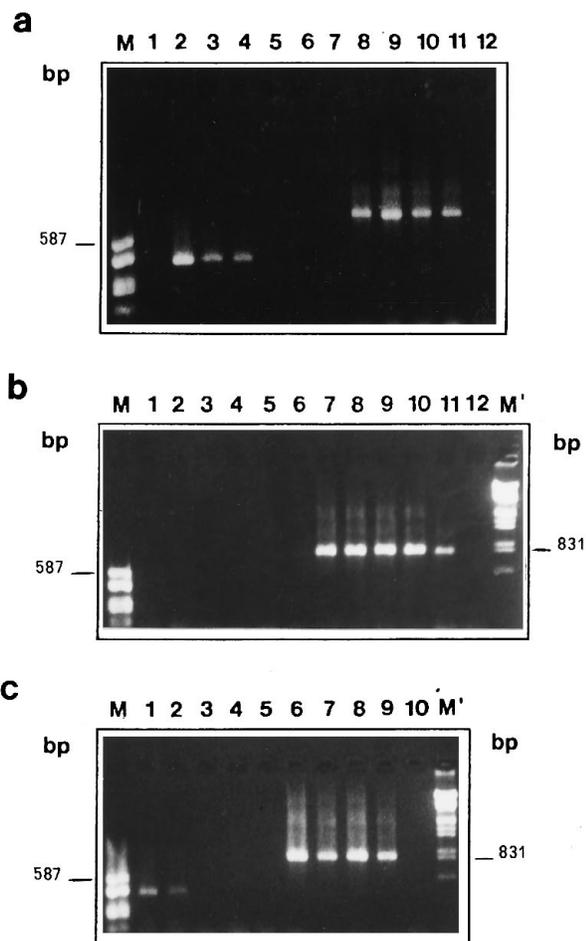
FIG. 3. PCR experiments. PCR assays were designed to look for BLOs at different stages of the life cycle of *Gigaspora margarita* and to rule out the possibility that the amplified and sequenced DNA fragment was derived from contaminating bacteria. In addition, other mycorrhizal fungi (a *Scutellospora* sp., *Acaulospora laevis*, and *Glomus mosseae*) were tested in these assays. (a) Agarose (2%) gel electrophoresis of PCR products amplified with primers BLOf and BLOr (lanes 1 to 6) or the eubacterial primers 704f and 1495r (lanes 7 to 12) on the following templates: washing solution (lanes 1 and 7), *Gigaspora margarita* spores (lanes 2 and 8), *Gigaspora margarita* external mycelium (lanes 3 and 9), clover roots colonized by *Gigaspora margarita* (lanes 4 and 10), clover roots (lanes 5 and 11), and no DNA (lanes 6 and 12). M, fragment size marker (pUC 18 DNA digested with *Hae*III). (b) Agarose (2%) gel electrophoresis of PCR products amplified with primers BLOf and BLOr (lanes 1 to 6) or the eubacterial primers 704f and 1495r (lanes 7 to 12) on the following control templates: *Tuber borchii* (lanes 1 and 7), *Agrobacterium tumefaciens* (lanes 2 and 8), *R. meliloti* GR4 (lanes 3 and 9), *Enterobacter agglomerans* (lanes 4 and 10), *Pseudomonas fluorescens* CHA0 (lanes 5 and 11), and no DNA (lanes 6 and 12). M and M′, fragment size markers (M, pUC 18 DNA digested with *Hae*III; M′, λ DNA digested with *Eco*RI-*Hin*dIII). (c) Agarose (2%) gel electrophoresis of PCR products amplified with primers BLOf and BLOr (lanes 1 to 5) or the eubacterial primers 704f and 1495r (lanes 6/10) on templates from the following: *Gigaspora margarita* spores (lanes 1 and 6), *Scutellospora* sp. spores (lanes 2 and 7), *Acaulospora laevis* spores (lanes 3 and 8), *Glomus mosseae* spores (lanes 4 and 9), and no DNA (lanes 5 and 10). M and M′, fragment size markers (M, pUC 18 DNA digested with *Hae*III; M′, λ DNA digested with *Eco*RI-*Hin*dIII).

significance of the *Burkholderia* population living in *Gigaspora margarita* remains unknown, its presence in fungi which are themselves obligately symbiotic organisms introduces a new level of complexity in mycorrhizal associations. We suggest that these bacteria are a stable component of the fungal cytoplasm and that they must be taken into account when considering the extent of microbial biodiversity in ecosystems.
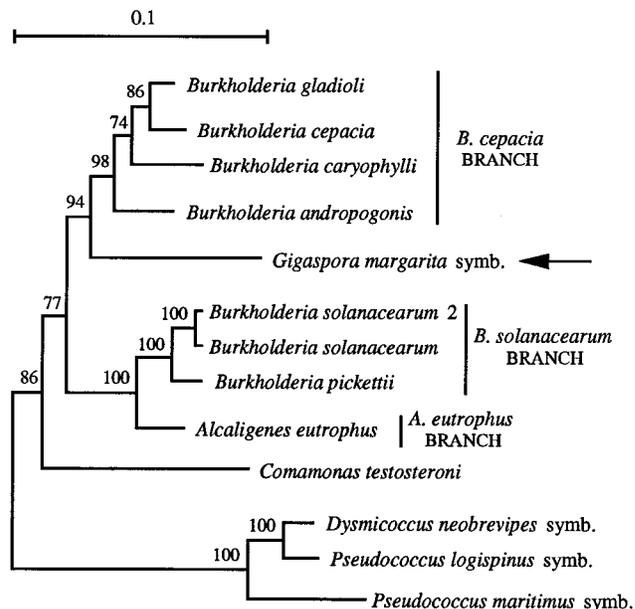

FIG. 4. Representative 16S rDNA-based tree (Kimura's correction; neighbor-joining method) illustrating the position of the *Gigaspora margarita* endosymbiont (symb.) (arrow) in relation to representatives of the group II pseudomonads (*B. cepacia* branch, *B. solanacearum* branch, *Alcaligenes eutrophus* branch) and other members of the β subclass of the proteobacteria. The endosymbionts of three insect species (*Dysmicoccus neobrevipes*, *Pseudococcus longispinus*, *Pseudococcus maritimus*) have been included for comparison. Insertions and deletions were taken into account. The numbers at the nodes are the bootstrap confidence values obtained after 100 replicates. The scale bar indicates distance in substitutions per nucleotide. The outgroup is *Escherichia coli* (data not shown).

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