

ORIGINAL ARTICLE

The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the *Mollicutes*

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Arbuscular mycorrhizal fungi (AMF) have been symbionts of land plants for at least 450 Myr. It is known that some AMF host in their cytoplasm Gram-positive endobacteria called bacterium-like organisms (BLOs), of unknown phylogenetic origin. In this study, an extensive inventory of 28 cultured AMF, from diverse evolutionary lineages and four continents, indicated that most of the AMF species investigated possess BLOs. Analyzing the 16S ribosomal DNA (rDNA) as a phylogenetic marker revealed that BLO sequences from divergent lineages all clustered in a well-supported monophyletic clade. Unexpectedly, the cell-walled BLOs were shown to likely represent a sister clade of the *Mycoplasmatales* and *Entomoplasmatales*, within the *Mollicutes*, whose members are lacking cell walls and show symbiotic or parasitic lifestyles. Perhaps BLOs maintained the Gram-positive trait whereas the sister groups lost it. The intracellular location of BLOs was revealed by fluorescent *in situ* hybridization (FISH), and confirmed by pyrosequencing. BLO DNA could only be amplified from AMF spores and not from spore washings. As highly divergent BLO sequences were found within individual fungal spores, amplicon libraries derived from *Glomus etunicatum* isolates from different geographic regions were pyrosequenced; they revealed distinct sequence compositions in different isolates. Our results show a vertically inherited, monophyletic and globally distributed lineage of endobacteria thriving in AMF cytoplasm. These bacteria split from their sister groups more than 400 Myr ago, colonizing their fungal hosts already before main AMF lineages separated. The BLO–AMF symbiosis can, therefore, be dated back at least to the time when AMF formed the ancestral symbiosis with emergent land plants.

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Symbiosis is central in the evolution of complexity, being crucial to the lifestyles of animals, plants, fungi and also prokaryotes (Hoffmeister and Martin, 2003). Many functions could only be evolved because of the intimate interaction and often interdependence of different species, as illustrated by endosymbioses, which gave rise to the eukaryotic organelles (Timmis *et al.*, 2004). Scaling up to the ecological level, symbiotically living organisms have key roles in most ecosystems (Moran *et al.*, 2008). Bacterial endosymbionts are widespread in animals, in particular in insects in which they—as ubiquitous and heritable genetic components—offer excellent models to investigate organelle evolution,

genome reduction and horizontal gene transfer (Moran *et al.*, 2008). In contrast, examples of endobacteria in fungi are limited to a few examples (Bonfante and Anca, 2009); among them a *Burkholderia* species living inside a strain of *Rhizopus* is responsible for fungal pathogenicity, and hence showing the interdependence of the bacterial–fungal alliance (Partida-Martinez and Hertweck, 2005).

Arbuscular mycorrhizal fungi (AMF), which are analyzed in this study, are long known to possess endobacteria in their cytoplasm. AMF belong to the *Glomeromycota*, a phylum known to be older than the *Ascomycota* and *Basidiomycota* (Schüßler *et al.*, 2001), and form symbiotic associations with >80% of land plants, in which both partners benefit from nutrient exchange. This association is commonly described as the result of co-evolution dating back to early Devonian times (Taylor *et al.*, 1995; Redecker *et al.*, 2000; Bonfante and Genre, 2008). As for insect endosymbionts, the presence of endobacteria inside

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AMF cytoplasm has long been documented by electron microscopy, which has distinguished two bacterial morphotypes. The first, restricted to a phylogenetically relatively young AMF family (*Gigasporaceae*), is rod shaped, related to *Burkholderia* and described as an uncultured taxon, *Candidatus Glomeribacter gigasporarum* (Bianciotto *et al.*, 2003). A fungal line cured from these endobacteria showed that they confer an ecologically relevant fitness to their fungal host (Lumini *et al.*, 2007). The other bacterial type has been detected inside AMF spores and hyphae colonizing plant roots sampled in the field. It is coccoid in shape and has been called 'bacterium-like organism' (BLO), as its identity is still obscure (MacDonald *et al.*, 1982; Scannerini and Bonfante, 1991; Schüßler *et al.*, 1994). When BLOs were first detected, knowledge of AMF phylogeny was limited and the fungi were classified only by their morphological features. As a consequence, BLO attribution to specific AMF taxa is uncertain and opens questions of whether BLOs in the fungal cytoplasm represent occasional re-infection by free-living bacteria, or whether they are a consistent feature.

To unambiguously identify the BLOs and assess their distribution in diverse members of the *Glomeromycota*, we analyzed AMF spores from 28 cultures, representing highly divergent lineages originating from four continents. We used confocal microscopy, fluorescence *in situ* hybridization (FISH) and electron microscopy to establish BLO localization in the AMF cytoplasm, together with sequencing and phylogenetic analysis on BLOs from single spores or groups. We report that BLOs live only within the fungal cytoplasm and that divergent, but monophyletic bacterial lineages, co-exist in an individual single spore. Unexpectedly, the cell-walled BLOs represent an old, formerly unknown bacterial group that likely falls within the clade harboring the cell wall-lacking *Mollicutes*. This finding opens new questions about the evolution of *Mollicutes*-related bacteria, their biotrophic lifestyle, the complexity of interphylum interactions and the symbiotic-genetic makeup of AMF.

Materials and methods

Spore manipulation

AMF cultures were obtained from different culture collections (Supplementary Table S1), in which they had been propagated for several generations either in pot soil cultures in the presence of a plant host or *in vitro* on root organ cultures (Cranenbrouck *et al.*, 2005). All steps requiring sterility were performed under laminar flow, and plastic material was sterile and DNA free. Spores were cleaned as described in Schwarzott and Schüßler (2001) and surface sterilized with 3% chloramine-T and 0.03% streptomycin.

DNA extraction and amplification

DNA was extracted from spores (either single or groups of five) as described in Lumini *et al.* (2007) with 10 µl 5 × HotStarTaq PCR buffer (Qiagen, Milan, Italy), or following Schwarzott and Schüßler (2001). The bacterial 16S ribosomal DNA (rDNA) was amplified by PCR with Phusion High-Fidelity DNA polymerase 2 × mastermix (Finnzymes, Espoo, Finland) and the general bacterial primers 16F27 (Bennasar *et al.*, 1996) and 1495r (Bandi *et al.*, 1994) nested with 16F27 and 1387R (Marchesi *et al.*, 1998). The final reaction mix contained 0.02 U µl⁻¹ Phusion polymerase, 1 × Phusion HF Buffer with 1.5 mM MgCl₂, 200 µM of each deoxynucleotide triphosphate and 0.5 µM of each primer. Thermal cycling conditions were: 5 min initial denaturation at 99 °C; 30–40 cycles of 10 s denaturation at 98 °C, 30 s annealing at 60 °C and 1 min elongation at 72 °C; and a 10 min final elongation. PCR products were TOPO cloned (Invitrogen, San Giuliano Milanese, Italy) and transformed into Top10 chemically competent *Escherichia coli* according to the manufacturers instructions. Colonies were screened for insert length by PCR. The PCR products were digested with the restriction endonucleases *AluI* and *RsaI* (Invitrogen) (1 U, 1 h at 37 °C) to produce restriction fragment length polymorphism profiles. A modified heat-lysis protocol (Ganguly *et al.*, 2005) was applied to extract plasmids. The plasmid inserts were sequenced from both ends on an ABI 3730 48 capillary sequencer with 50 cm capillary length using BigDye v3.1 sequencing chemistry (Applied Biosystems, Darmstadt, Germany).

Bioinformatic analyses

Sequences were assembled and curated using Seqassem (Sequentix, Klein Raden, Germany) and aligned with the SILVA 16S RNA database (Pruesse *et al.*, 2007), version SSURef_release96, using ARB (Ludwig *et al.*, 2004). Phylogenetic trees were inferred with the PHYLIP package (Felsenstein, 1989), MrBayes (Huelsenbeck and Ronquist, 2001) and RaxML (Stamatakis *et al.*, 2008). Only topologies are shown, which are supported by at least two of the three phylogenetic analysis methods used (neighbor joining, maximum likelihood and Bayesian) with >50% bootstrap or >0.5 posterior probability values. Others are collapsed to polytomies. Dashes instead of numbers indicate that the topology was not supported in the respective analysis (Figure 3). Pairwise distances between sequences types were estimated with ClustalW (Larkin *et al.*, 2007).

Pyrosequencing

A metagenomic approach was adopted for the 454-based experiments; as BLOs are so far uncultured, the starting material was fungal spores. For the construction of amplicon libraries from endobacteria and bacteria associated with the surface of *Glomus*

versiforme Att 475–45, we used three types of samples: (1) washed spores, (2) spores sonicated in 10 µl sterile DNA-free water for 2 min and washed three times afterwards and (3) the water remaining after the sonication. Spores of *G. etunicatum* culture Att 239–4 were decontaminated as in sample 2. Spores from *G. etunicatum* isolates MUCL 47650, CA-OT126-3-2 and CA-OT-135-4-2 were collected from *in vitro* culture and washed twice. Each sample combined five spores or the equivalent water and we used three biological replicates. DNA was extracted as in Schwarzott and Schüßler (2001), and amplified by PCR with Phusion HF Mastermix (as described above) with modified versions of the primers 967F (5'-GCCTCCCTCGCGCCATCAGNNNCRACGCGNA GAACCTTACC-3') (Sogin *et al.*, 2006) and 1495r (5'-GCCTTGCCAGCCCGCTCAGCTACGGYTACCTG TTACGAC-3') (Bandi *et al.*, 1994), chosen to construct tagged fusion primers (Supplementary Table S3, SI). Thermal cycling conditions were: 5 min initial denaturation at 99 °C; 30 cycles of 10 s denaturation at 98 °C, 30 s annealing at 57 °C and 1 min elongation at 72 °C; and a 10 min final elongation. Each DNA extract was amplified with its respectively tagged fusion primer in five independent PCRs. Products were pooled and purified with the AMPure kit (Agencourt, Beckman Coulter S.p.A, Milan, Italy), quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Euroclone S.p.A., Milan, Italy) and combined equimolar. Product quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Cernusco, Italy) and sent to BMR Genomic (Padova, Italy) for emulsion PCR and pyrosequencing on a GS FLX sequencer (Roche, Mannheim, Germany).

Bacterial rDNA sequences from *G. etunicatum* and *G. versiforme* metagenomic DNA were quality trimmed (quality cutoff set to 20, omitting sequences < 150 b), sorted to the respective sample through the tag sequences, and aligned and clustered using the pyrosequencing pipeline provided by the ribosomal database project (Cole *et al.*, 2009). Operational taxonomic units were classified with the ribosomal database project classifier. In addition, a dereplicated subset of sequences (one sequences per sample per operational taxonomic unit recovered at the 3% sequence divergence level) was aligned with SINA (the SILVA web aligner, <http://www.arb-silva.de/aligner/>; Pruesse *et al.*, 2007), imported and inserted into the 'All-Species Living Tree' alignment (Yarza *et al.*, 2008) with ARB. This alignment had been supplemented with the nearly full-length 16S rDNA sequence data of BLOs before use.

Morphological detection of BLOs

The samples were observed with a confocal laser scanning microscope (CLSM, Leica TCS SP2, Leica Microsystems Srl, Milan, Italy) exciting at 488 and 543 nm. A 40 × long-distance water immersion objective (HCX APO N.A. 0.80) and a 63 × water

immersion objective (HCX PL APO N.A. 1.20) were used. Images were taken sequentially at each excitation wavelength. Pseudocolors for emission wavelength were green for 500–530 nm (fluorescein isothiocyanate and SYTO BC) and red for 550–580 nm (Cy3) and 605–635 nm (propidium iodide). To rapidly screen AMF spores for endobacteria, they were stained with the dye mixture SYTO BC (1–5 µM; Molecular Probes, San Giuliano Milanese, Italy), known to penetrate intact living bacterial cells. Fungal nuclei and dead bacteria were counterstained with propidium iodide (30–90 µM). Samples were incubated for 15 min at room temperature before spore crushing and observation.

FISH experiments

Before fixation, *G. versiforme* Att 475–45 spores were vortexed for 2 to 3 min to clean their surface, and washed three times with ultrapure water and once with phosphate-buffered saline. Spores were fixed in 3% formaldehyde buffered with phosphate-buffered saline (Amann *et al.*, 1990) incubated at 4 °C for 3 h or 6 h, washed three times in the saline, suspended in 50% ethanol in the saline, and stored at –20 °C until use.

Oligonucleotide probes were purchased from Sigma-Aldrich (Milan, Italy) and Thermo Fisher Scientific GmbH (Ulm, Germany), 5'-end labeled with Cy3 or fluorescein isothiocyanate. The eubacterial probe EUB338I (Amann *et al.*, 1990) was used as a general probe, and Apis2Pa (Moran *et al.*, 2005), which targets the genus *Buchnera*, was used as a negative control to detect nonspecific binding. Specific oligonucleotide probes were designed with ARB. They matched bacterial sequences amplified from *Glomus* group B (*Glomerales*) and *Diversisporales* and showed a minimum of three centrally located mismatches to all other sequences in the ARB 16S database. The specificity of BLOgrBC (5'-GCCAAT CCTACCCTTGTC-3') was tested empirically. Fungal spores were immobilized on 10-well microscope slides (6 mm Ø each well, Structure Probe, West Chester, PA, USA) with a drop of 0.5% agarose (Sigma-Aldrich) and dehydrated in 50%, 75% and 100% ethanol. Spores were crushed to allow penetration of the probes into the cytoplasm during hybridization, as described in Bertaux *et al.* (2005). The probes were hybridized at a stringency of 35% formamide (Sigma-Aldrich).

Microscope slides were mounted with SlowFade Antifade kit component A (Molecular Probes). The hybridization to EUB338I and BLOgrBC was repeated 14 times, corresponding to a total of 40 *G. versiforme* spores.

Transmission electron microscopy

To allow fixative penetration into the spore, spore walls of surface-sterilized *G. versiforme* Att 475–45 spores were incised with a fine syringe needle

(U100 Insulin ACCU-FINE; Roche, Monza, Italy). These spores were immediately transferred to 50 mM sodium cacodylate buffer (pH 7) containing in 2.5% (v/v) glutaraldehyde and then processed as described in Bianciotto *et al.* (2003) and Lumini *et al.* (2007).

Ultrathin sections (70 nm) were cut with an ultramicrotome (Ultracut; Reichert and Jung, Vienna, Austria) counterstained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Philips CM10, Philips Medical Systems, Eindhoven, The Netherlands). In all, 10 individual spores were examined.

Results

BLOs are present in diverse AMF families

Spores from 28 cultures, including *in vitro*-grown root organ cultures and pot cultures, were studied. They represent 16 phylogenetically characterized species belonging to diverse *Glomeromycota* lineages and originating from four continents (Figure 1; Supplementary Table S1, SI). After staining with the fluorescent dyes SYTO BC and propidium iodide, the BLOs were observed within the spore cytoplasm as green fluorescent spots approximately 500 nm in diameter, whereas fungal nuclei appeared red (Figure 2a). BLOs were consistently found in 19 of the cultures and were associated with 11 of the 16 AMF species tested, being present in members of the *Ambisporaceae* and

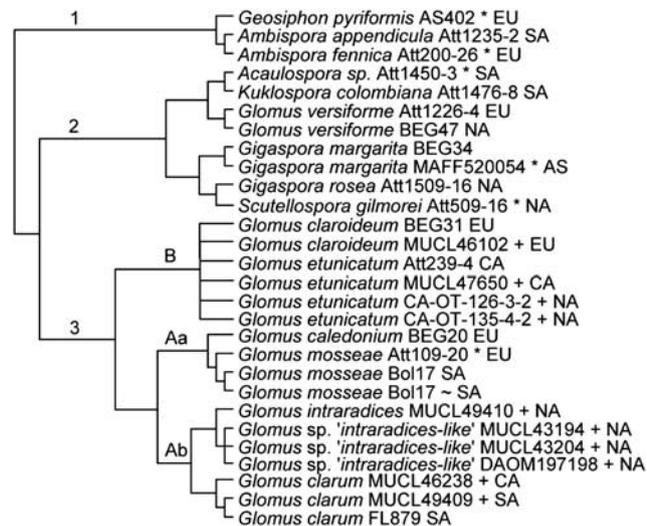


Figure 1 Scheme of taxonomic relationships between AMF isolates and cultures used for molecular and cell biological investigations. The order level is indicated: *Archaeosporales* (1), *Diversisporales* (2) and *Glomerales* (3). *Glomerales* can be further split into the *Glomus* group Aa, Ab and B. The fungal species is followed by the respective culture name, and * (isolates in pot culture), + (isolates *in vitro* on root organ culture (ROC)) or ~ (culture *in vitro* on whole plants (WPC)), and other cultures are multispore cultures (not isolates). The geographical origins are in Asia (AS), Europe (EU), North America (NA), Central America (CA) and South America (SA).

Geosiphonaceae (*Archaeosporales*, an ancient AMF lineage), *Glomus* group A and *Glomus* group B (corresponding to two families in the *Glomerales*), and *Diversisporaceae*, *Gigasporaceae* and *Acaulosporaceae* (*Diversisporales*) (Figure 1; Supplementary Table S1, SI).

BLOs are related to the Mollicutes

Bacterial 16S rDNA was amplified from surface-sterilized or *in vitro*-cultured AMF spores using general bacterial primers. A set of BLO-specific primers was then developed, based on the sequence data obtained. In total, 107 distinct sequence types were obtained from 17 of the 28 cultures, including members of the *Glomerales* (*G. caledonium*, *G. mosseae*, *G. claroideum* and *G. etunicatum*), *Diversisporales* (*Gigaspora margarita*, *Scutellospora gilmorei* and *G. versiforme*) and the *Archaeosporales* (*Ambispora appendicula*, *A. fennica* and *Geosiphon pyriformis*). BLO 16S rDNA could not be amplified from an *Acaulospora* sp. and *Kuklospora colombiana* (*Acaulosporaceae*, *Diversisporales*), notwithstanding the morphological evidence of bacterial structures by fluorescent staining.

All sequences clustered in a well-supported monophyletic clade that was not closely related to any described bacterial group. BLOs therefore represent a new, undescribed bacterial higher taxon that, although there is a distinct cell wall (Figure 2b), forms a subclade within the wall-less *Mollicutes* (Ludwig *et al.*, 2008) (Figure 3) likely being a sister clade to the *Entomoplasmatales* and *Mycoplasmatales*.

Highly divergent BLO sequences exist within a single AMF spore

Within the monophyletic BLO clade (Figure 3), highly variable 16S rDNA BLO sequences were found, showing up to 20% intraspore sequence divergence even within single AMF spores. BLO sequences from an AMF species formed from one (*A. fennica*) up to six divergent subclades (*G. claroideum*). The branches in the basal part of the tree included sequence types from the *Archaeosporales*, *Diversisporales* and *Glomerales*, and their topological relationships could not be resolved. In contrast, the more terminal part of the tree was better resolved. It only contained bacterial sequences from *Glomerales* and *Diversisporales* (Supplementary Figure S1, SI). Here, BLO sequences stemming from closely related fungi clustered together, indicating co-evolution with their hosts.

BLOs are located within the AMF cytoplasm

To unambiguously show that BLOs are located in the cytoplasm of glomeromycotan fungi, *G. versiforme* (Att 475–45) was selected because of easy production and handling of spores, low degree

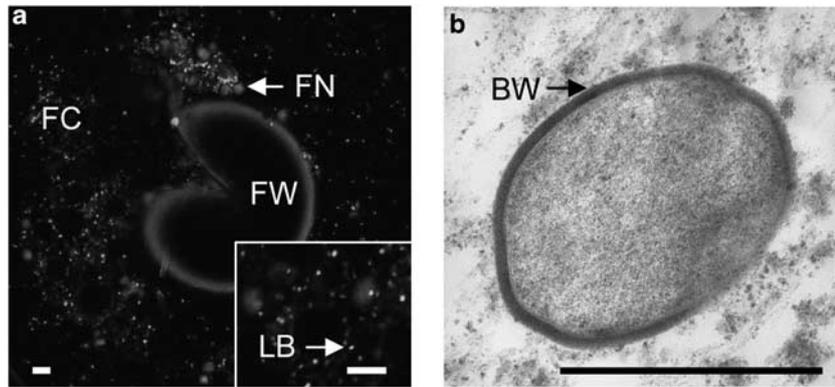


Figure 2 Bacterial structures inside AMF. (a) Bacterial and fungal structures in the fungal cytoplasm (FC) of a manually crushed *G. etunicatum* spore, stained with the fluorescent dyes SYTO BC and propidium iodide (PI). Inset: enlarged portion of cytoplasm. Living bacteria (LB) appear as coccoid structures in green, whereas fungal nuclei (FN), dead bacteria and the fungal spore wall (FW) appear in red. Scale bars, 10 µm. (b) Electron micrograph of a BLO inside *G. versiforme* cytoplasm, showing a prominent Gram-positive cell wall (BW). Scale bars, 0.5 µm. The color reproduction of this figure is available on the html full text version of the manuscript.

of cytoplasm autofluorescence and its long-standing use as a model fungus in arbuscular mycorrhizal research (e.g., Harrison and Buuren, 1995). The 16S rDNA sequences obtained in our work allowed us to develop specific probes for FISH. The new BLO-specific probe BLOgrBC colocalized with the widely used general bacterial probe EUB338I within the *G. versiforme* cytoplasm (Figure 4) in which it labeled coccoid structures (Figure 4, compare with Figure 2a). The FISH signal was not detected in any free-living bacteria or those associated with the spore surface (data not shown). FISH also indicated that the structures stained by SYTO BC were indeed bacteria.

Electron microscopy of the same *G. versiforme* culture confirmed the presence of coccoid bacteria in the fungal cytoplasm. They were 250–500 nm in size, possessed a homogeneous, Gram-positive type cell wall and, unlike *Candidatus Glomeribacter*, were not surrounded by a fungal membrane (Figure 2b).

To confirm the cytoplasmic localization of the BLOs we used the 454 GS-FLX technology (Margulies *et al.*, 2005) to sequence 16S rDNA amplicon libraries. The metagenomic DNA was derived from either (1) five washed spores, (2) five sonicated and washed spores or (3) the water in which the five spores were held during sonication, with three biological replicates each. A total of 1125 high-quality sequences were obtained. Sequences detected from the sonicated water were considered as derived from spore surface-associated bacteria. The results showed that sonication did not remove all surface bacteria but the percentage of non-BLO sequences associated with the surface of sonicated spores was lower than for spores only washed (Figure 5; Supplementary Figure S2, SI). They belonged to bacterial groups already reported as associated with AMF spores (reviewed in Bonfante and Anca, 2009) and in studies of soil biodiversity (Roesch *et al.*, 2007; Elshahed *et al.*, 2008; Kielak *et al.*, 2008). In contrast, BLO sequences were

exclusively obtained from washed and sonicated spores but never from sonicated water. This points to their presence inside fungal spores and more importantly shows that BLOs were not detected as free-living bacteria. Sequences related to those of BLOs have been reported in some recent studies. With the exception of one that was shown to be associated with *Gigaspora margarita* (Long *et al.*, 2009), all the other sequences are annotated as environmental and have been retrieved in studies on soil bacterial diversity, from tree and grassland environments (Dunbar *et al.*, 2002; Elshahed *et al.*, 2008; Hansel *et al.*, 2008; Lesaulnier *et al.*, 2008; Cruz-Martinez *et al.*, 2009). Most likely, these sequences originated from BLO-harboring AMF present in the samples, either as spores or, hyphae in the soil or in root fragments.

BLO rDNA sequence variability: another source of genetic variability in arbuscular mycorrhizal fungi
The model AMF *Glomus* sp. ‘intraradices-like’ DAOM197198, whose genome is currently under sequencing, has been indicated to contain multiple, polymorphic genomes (Hijri and Sanders, 2005; Martin *et al.*, 2008). In contrast, the mitochondrial genome of this fungus and its close relatives (Lee and Young, 2009) including the mt-rDNA (Börstler *et al.*, 2008), is nearly invariable. For isolates of *G. intraradices* and the species represented by *Glomus* sp. DAOM197198, exceptionally variable nuclear rDNA regions were reported with up to >20% intrasporal sequence divergence in the internal transcribed spacer region (Stockinger *et al.*, 2009). A similarly high internal transcribed spacer variability was recently also reported for AMF from other phylogenetic clades (Stockinger *et al.*, 2010). Unexpectedly, a very high variability (up to 20% intrasporal sequence divergence) was found between BLO 16S rDNA sequences attributed to a single fungal culture and even to single spores

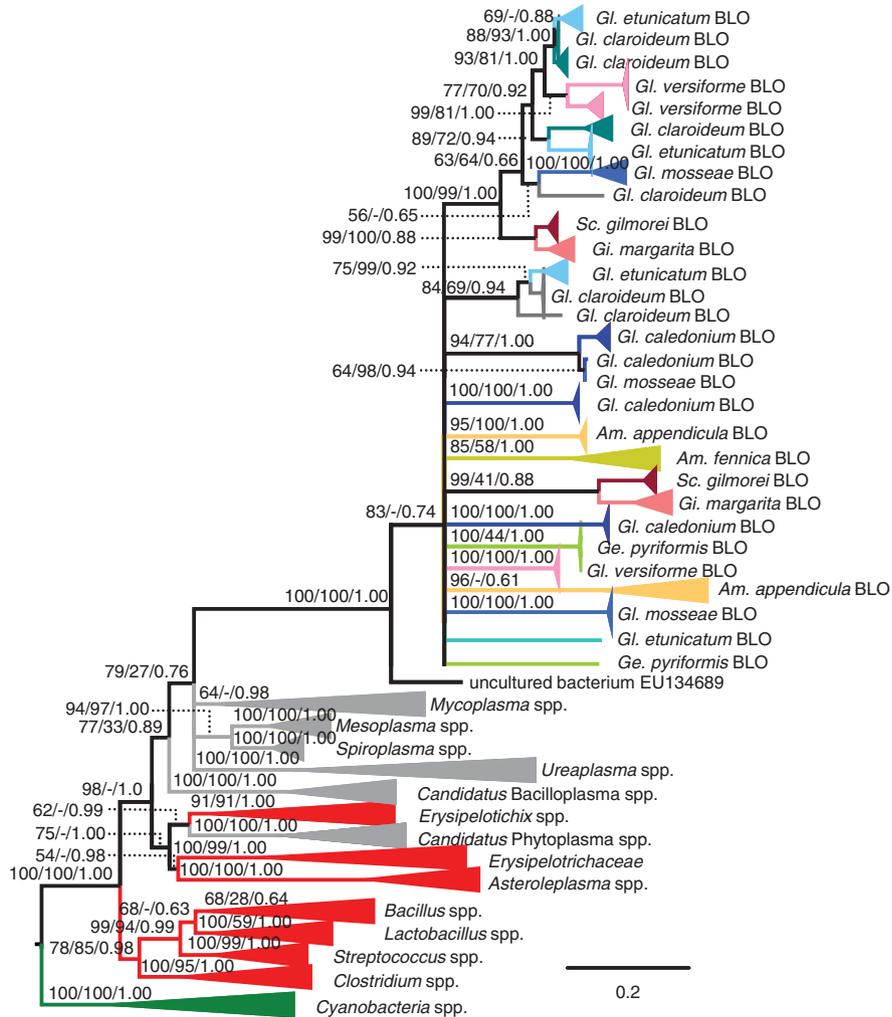


Figure 3 Phylogenetic tree based on the 16S rDNA, showing the position of BLO sequences within the clades of *Mollicutes* (red) and *Firmicutes* (gray); *Cyanobacteria* (dark green) sequences are used as outgroup. BLO sequences are grouped and coded in different colors according to the associated AMF species. Shades of the same color are applied to sequences from the same AMF family. One to six subclades of BLO sequences were retrieved per AMF species. Where the branching pattern is resolved, BLO sequences from closely related AMF are found to group together. Support values given are from maximum likelihood/neighbor joining/Bayesian analyses. Dashes instead of numbers imply that the topology was not supported in the respective analysis. The organization within the clades is shown in Supplementary Figure S1 a and b (SI).

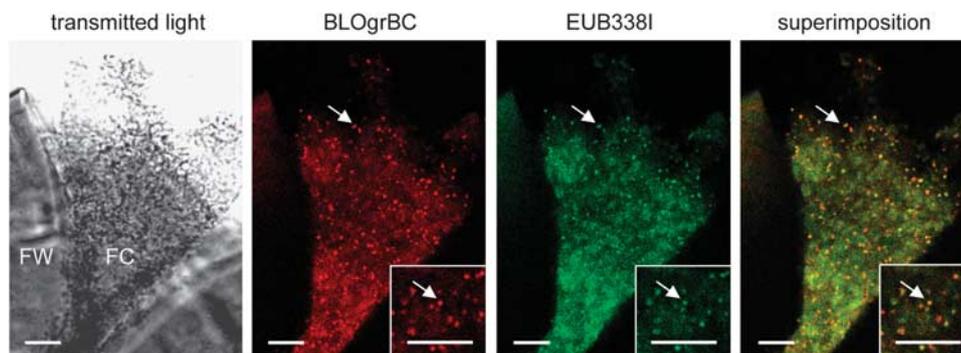


Figure 4 FISH on a crushed *G. versiforme* spore. The location of the spore wall (FW) and the squeezed out cytoplasm (FC) is shown in transmitted light (left side). Bacteria are seen as coccoid fluorescent spots in the fungal cytoplasm (indicated by arrows), their 16S rRNA hybridized to the BLO-specific probe BLOgrBC (red) and to the general eubacterial probe EUB338I (green). Both probes label the same structures, as seen in the superimposition of the signals (right side). The insets show part of the cytoplasm enlarged. Scale bars, 10 µm.

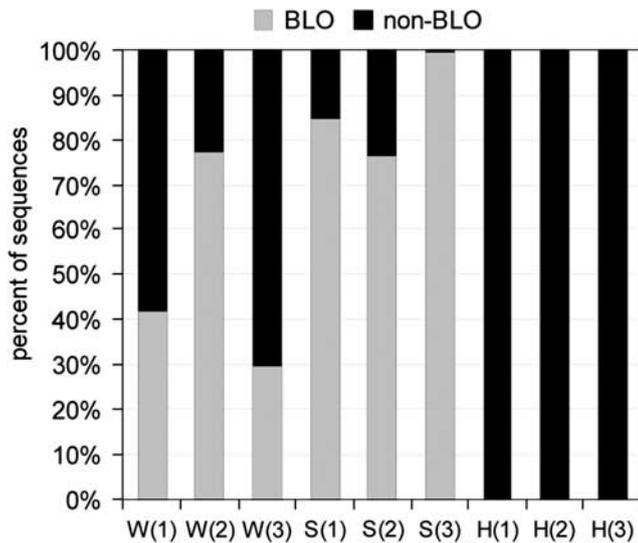


Figure 5 Sequences recovered from 16S rDNA amplicon libraries of *G. versiforme* are classified as BLOs (gray) or non-BLOs (black); the percentage of sequences that can be attributed to either group is shown. The metagenomic DNA was derived from washed spores (W), sonicated and washed spores (S) or the water in which the spores were sonicated (H), with three biological replicates each (1, 2 and 3). Spores were checked and remained intact after sonication; therefore, bacterial sequences retrieved from the sonicated water were considered as originating from surface-associated bacteria. Sonication did not remove all surface bacteria, but the percentage of non-BLO sequences associated with the surface of sonicated spores was lower than for spores only washed, and no BLO sequences were recovered from (H).

in the present study, although not all these variations could be recovered from each culture. To understand whether this was due to insufficient sampling density, amplicon libraries of *G. etunicatum* spores were 454 GS-FLX pyrosequenced.

G. etunicatum was chosen because four cultures were available for comparison. Three of these were single spore isolates established as root organ cultures (CA-OT-126-3-2, CA-OT-135-4-2 and MUCL47650), and one was propagated in pot (Att 239-4) (Figure 1; Supplementary Table S1, SI). Each library was again generated from five spores, with three biological replicates each, leading to 2827 sequences. To define phylotypes, all sequences with $\leq 3\%$ divergence were clustered. Only one of the resulting 20 BLO phylotypes was present in all four *G. etunicatum* cultures, one was exclusively detected in the biological replicates of MUCL47650 and Att 239-4, one only in the biological replicates of CA-OT-135-4-2, whereas several other phylotypes could be attributed to both CA-OT-126-3-2 and CA-OT-135-4-2 isolates, both originating from California (Table S2, SI). Thus, pyrosequencing confirmed that different phylotype compositions do indeed exist between isolates of the same AMF species, and highlighted that the BLO sequences represent the vast majority (89.8%) of the bacterial sequences recovered from cleaned spores. When the *in vitro*

cultures, expected to be free of contaminating bacteria, were considered, BLO sequences represented even 93.6%. Although the BLO phylotypes were present throughout all biological replicates of a sample, the remaining non-BLO sequences were highly diverse and variable in between replicates, indicating contaminants acquired during sample preparation. However, the percentage values have to be interpreted with care, as sequences may be artificially replicated during pyrosequencing (Gomez-Alvarez *et al.*, 2009).

Discussion

Mycorrhizas, the symbioses established by most land plants and soil fungi, are often described as the result of tripartite interactions, as many bacteria are known to be associated as free-living helper microbes (Frey-Klett *et al.*, 2007). In contrast, existence of true endobacteria in mycorrhizal fungi has been convincingly shown only for the betaproteobacterium *Candidatus Glomeribacter gigasporarum*, whose presence is restricted to members of the family *Gigasporaceae* (Bonfante and Anca, 2009). In this study we show that the Gram positive endobacteria (the BLOs) live in the cytoplasm of many AMF lineages, and that their occurrence is not a sporadic event. BLOs are present in *Glomus* group A and B of the *Glomerales*, in *Diversisporales* and in *Archeosporales*, covering diverse evolutionary lineages in the AMF. Interestingly, they were not detected in *Glomus* sp. 'intraradices-like' DAOM197198, *G. intraradices* and *G. clarum*, which are all closely related and belong to the subclade *Glomus* group Ab (Schwarzott *et al.*, 2001).

The AMF-BLO sequences cluster within those of the *Mollicutes*, despite the presence of a distinct cell wall and represent a new bacterial higher taxon. The AMF cultures that tested positive for BLOs originated from four continents and represent diverse families, including more basal evolutionary lineages. This indicates that BLOs already colonized AMF before the split of those early diverging AMF lineages. As a consequence, and from the phylogenetic tree topology, the BLOs must have been living in AMF for more than 400 Myr. Because many of the AMF cultures studied here were maintained in *in vitro* root organ cultures, we also obtained evidence that BLOs are vertically transmitted, similar to many endobacteria living in insects (Baumann & Moran, 1997) and *Candidatus Glomeribacter gigasporarum* inside AMF of the *Gigasporaceae*.

Theory predicts that vertically transmitted symbionts, for example, in insects, should offer some benefit to the host as they are maintained by it (Brownlie and Johnson, 2009). Even if, at the moment, we have no clue to BLO function, we can infer that BLOs likely confer fitness on their fungal hosts. Electron microscopy shows that BLOs

are directly embedded in the fungal cytoplasm, without a surrounding host membrane, indicating a high level of compatibility or an ancient interaction.

Taken together, our results show that AMF-BLOs are pan-global, vertically inherited, monophyletic, ancient and, so far, uncultured and most likely *Mollicutes*-related endobacteria. Although the 16S phylogenies place BLOs with *Mollicutes* rather than with *Firmicutes*, the exact placement of the BLO clade should currently be treated with caution. The relationship between *Mollicutes*, which belong to the *Tenericutes*, and the *Firmicutes* is in fact not clearly solved (Ludwig *et al.*, 2008; Battistuzzi and Hedges, 2009). To unambiguously resolve the relationships within these phyla and then attribute BLOs to one of them, data from other genes will be required. Nevertheless, the finding that the BLOs, *Entomoplasmatales* and *Mycoplasmatales* likely share a common ancestor raises questions about the biology and evolution of the *Mollicutes*. A common characteristic is their endosymbiotic or parasitic lifestyle (Johansson and Pettersson, 2002). The intracellular status of the AMF-BLOs supports the concept that the ancestors of *Mollicutes* had already evolved mechanisms to exploit intracellular or symbiotic niches, as showed by the present-day species in the genera *Phytoplasma* and *Mycoplasma*, which are widespread parasites of animals and plants. Intriguingly, the BLO cell wall, absent from other members of the *Mollicutes*, indicates that BLOs maintained a trait that sister clades have lost, after the divergence of *Mollicutes* from their *Firmicute*-related ancestors, between 600 and 2000 Myr (Maniloff, 2002; Battistuzzi and Hedges, 2009). The presence of a cell wall and the phylogenetic branching of the BLOs support the concept of an ancestral association between BLOs and AMF. Because AMF themselves have been involved in symbioses with plants since Devonian times (Taylor *et al.*, 1995; Redecker *et al.*, 2000), our findings open new questions on the complexity and age of multiple and symbiotic inter-phylum interactions.

Lastly, in the broader context of eukaryotic cell evolution, the plant-symbiont AMF have been unique up to now; they maintain an extraordinarily high polymorphism in their genomes (Martin *et al.*, 2008), as reflected by intrasporal nuclear rDNA variability (Stockinger *et al.*, 2009, 2010), likely resulting in a population of heterokaryotic nuclei. The variability of BLO rDNA sequences now indicates a similar variability for the AMF-associated endobacterial population, even within one fungal spore. BLOs seem to be widespread not only within the AMF maintained in culture, as investigated in this study, but also in environmental samples, as is emerging from ultrastructural observations (e.g., Ligrone *et al.*, 2007). For this reason, their pan-global distribution and genetic variability, apparently associated with avoidance of a bottleneck, add a further level of interest to the still enigmatic AMF genomes.

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