Bacteria Associated with Spores of the Arbuscular Mycorrhizal Fungi

Glomus geosporum and Glomus constrictum

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Spores of the arbuscular mycorrhizal fungi (AMF) Glomus geosporum and Glomus constrictum were harvested from single-spore-derived pot cultures with either Plantago lanceolata or Hieracium pilosella as host plants. PCR-denaturing gradient gel electrophoresis analysis revealed that the bacterial communities associated with the spores depended more on AMF than host plant identity. The composition of the bacterial populations linked to the spores could be predominantly influenced by a specific spore wall composition or AMF exudate rather than by specific root exudates. The majority of the bacterial sequences that were common to both G. geosporum and G. constrictum spores were affiliated with taxonomic groups known to degrade biopolymers (Cellibrio, Chondromyces, Flexibacter, Lysobacter, and Pseudomonas). Scanning electron microscopy of G. geosporum spores revealed that these bacteria are possibly feeding on the outer hyaline spore layer. The process of maturation and eventual germination of AMF spores might then benefit from the activity of the surface microorganisms degrading the outer hyaline wall layer.

Arbuscular mycorrhizal fungi (AMF) play a key role in facilitating nutrient uptake by crops in low-input farming systems, a prerequisite to maintain sufficient productivity under these conditions (3). AMF spores provide a long-term reservoir of inoculum and are the only AMF propagules that can be identified to the species level (33). The spore wall of Glomus geosporum is composed of three layers: an outer hyaline layer that decays until it sloughs off, leaving a granular surface; a laminated yellow-brown to orange-brown middle layer; and a more rigid inner layer that is often adherent to the middle layer (14). The thin hyaline layer is composed mainly of chitin (32) and has been found to be often colonized by microorganisms in several Glomus species (5). The Glomus constrictum spore wall is composed of only two layers, a decomposing outer hyaline layer that is absent in older spores and a rigid, laminated orange-brown to reddish-black dark layer (14).

An optimal colonization of plant roots, particularly in disturbed habitats such as agricultural fields, depends not only on the presence of extraradical hyphae or mycorrhizal root debris but, mainly, on the survival and well-timed germination of AMF spores in the soil. This process can be altered by various abiotic and biotic factors, in particular by the association with soil microorganisms (38). Indeed, some bacterial populations, called mycorrhiza helper bacteria, have beneficial effects on AMF growth not only by improving mycorrhizal root colonization and stimulating extraradical hyphal growth but also by facilitating AMF spore germination (11, 12). The latter effect has been shown for Actinomycetes (2, 7, 22), Pseudomonas and Corynebacterium (21), and Bacillus (38) spp.

Bacteria associated with AMF spores colonize mainly the outer wall layer and rarely penetrate into the inner layers (5, 9, 19, 36). Nevertheless, some bacteria have been found in the cytoplasm of AMF spores (4, 18). The role of AMF spore-associated bacteria is not clear. They could stimulate spore germination by eroding spore walls (9, 19), by producing stimulatory compounds such as CO2 and other volatiles (7), or by influencing AMF phosphorus acquisition (30).

Root exudation could enhance spore germination by stimulating the growth of bacteria beneficial for AMF (21). However, since the quantity and composition of exudates differ from one plant to another (17), different bacterial populations could be stimulated, depending on their preference for distinct plant exudates.

In most of the previous studies on spore-associated microorganisms, the bacteria were isolated upon culturing. However, bacteria not cultivable on ordinary media could represent a significant part of the bacterial community associated with AMF spores. Indeed, only a small fraction (1 to 10%) of the total bacterial community is cultivable (1). Direct molecular approaches that avoid a cultivation step give a broader picture of bacterial communities. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of the 16S rRNA gene permits fingerprinting of the dominant bacteria of a given sample (10, 23). The detection of populations representing as little as 0.1 to 1% of the target organisms is feasible. In the present study, the bacterial community associated with spores of Glomus geosporum BEG 18 and G. constrictum BEG 19 was assessed with PCR-DGGE analysis. In order to find out whether specific root exudates or, rather, the fungal species determine the bacterial community structure, spores of the two Glomus species were harvested from both pot cultures with Plantago lanceolata (Plantaginaceae) and pot cultures with Hieracium pilosella (Asteraceae) as host plants.
MATERIALS AND METHODS

Mycorrhizal inoculum. The AMF used in this study were Glomus geosporum (BEG 18) and Glomus constrictum (BEG 19), originating from the same calcareous grassland at Nenzlingen, Switzerland (35). The single-spore-derived cultures were maintained by subculturing in pots under the same conditions, using Plantago lanceolata and Heracium pilosella as host plants. The seeds were purchased from FENACO (Winterthur, Switzerland). The growth substrate was TerraGreen-sand-loess (2:2:1) (TerraGreen; Weninger GmbH, Telfs, Austria).

Experimental setup. Twelve 1-liter plastic pots were filled with sterile substrate composed of TerraGreen, quartz sand, and loess (5:4:1) and moistened with water. In each pot, a small hole was drilled, in which a teaspoon of mycorrhizal inoculum was placed. On top of the inoculum, a few seeds of Heracium pilosella or Plantago lanceolata were sown and covered with sterile quartz sand. The Tropf-Blumat watering system was installed, and the cultures were grown in a greenhouse with ambient natural light and temperature conditions and irrigated with deionized water by using an automated watering system (Tropf-Blumat; Weninger GmbH, Telfs, Austria).

Sampling. Six 15-ml soil cores were sampled from each pot after 170 days of growth in the case of H. pilosella and 247 days of growth in the case of P. lanceolata in order to obtain a sufficient amount of spores. The soil cores were wet sieved through 250- and 63-μm meshes. The residue gained from the 63-μm mesh sieve was centrifuged at 900 × g for 2 min in a sterile plastic tube, and the supernatant was discarded. The pellet was air dried for 15 min. The DNA was resuspended in 50 μl 10 mM Tris-HCl, pH 7.5. The V3 region of the DNA was then amplified according to the PCR protocol described above. Again, the amplified products were loaded on a DGGE gel to improve DNA yield and check band purity. If the band on this second gel matched the previously selected one, it was cut out, purified, and reapplied the same way. The amplified products were then purified with the NUCLEOETRAP-CR kit (Macherey-Nagel, Düren Germany) according to the manufacturer’s protocol. The DNA fragments were ligated using the pGEM-T vector system (Promega), following the protocol of the manufacturer. Transformation was performed by electroporation using the Bio-Rad Gene Pulser XCell and PC module into E. coli XLI-Blue. The transformed bacterial cells were then plated onto Luria-Bertani (LB) agar containing ampicillin (150 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (0.1 mM) and IPTG (isopropyl-β-D-thiogalactopyranoside) (0.2 mM). Plasmids were recovered from white colonies by using the NucleoSpin plasmid kit (Macherey-Nagel) according to the manufacturer’s protocol. The resulting DNA fragments were sequenced by Macrogen Corp., South Korea. Three clones per band were sequenced, and only the bands having similar sequences in two out of these three clones are presented in Results. The 16S RNA gene sequences were aligned using the ClustalX software (34), and the phylogenetic trees were constructed using the neighbor-joining method (31) with the NJplot software (ftp://phyl Dirk -lyon.fr/pub/mol_phylgeny/njplot) (25). The topology of the distance tree was tested by resampling data with 100 bootstrap (8) to provide confidence estimates for tree topologies.

SEM analysis. The spores were fixed using 1% OsO4 and air dried. After being coated with gold, the samples were examined with a Phillips XL 30 scanning electron microscope with an acceleration voltage of 10 kV.

Statistical analysis. To analyze the relationships between the DGGE patterns of the different samples, correspondence analysis (CA) was used. This ordination method is adapted to analyze presence/absence or abundance data tables and is well suited for populations with unimodal distributions along environmental gradients (10). To perform the CA, a data matrix was composed of rows representing the condition culture replicate and columns of species representing a DGGE band position along the vertical gel gradient. The relative abundance of a species in a sample corresponded to the DGGE band’s relative intensity with regard to the sum of all band intensities in a pattern. The CA was then applied on the basis of numerical data matrices converted using the program Progiciel R (16). From the association matrix obtained, the characteristic values associated with the characteristic vectors were calculated using a multi-dimensional dispersion cloud of the data with the Canoco 4.0 software (Microcomputer Power, Ithaca, N.Y.). Variation partitioning analysis (6) enables display of the variability of patterns constrained by the factors of interest. Therefore, this analysis was used to display the contributions of an AMF species or plant species to the bacterial community profiles. The significance of the results was tested with the Monte Carlo permutation test. Variation partitioning analysis was performed with the software R (26).

Sequence analysis. The same DGGE band sequences were submitted to the EMBL nucleotide sequence database and assigned accession no. AJ864379 to AJ864393.

RESULTS

The homogeneity among replicates of DGGE patterns of bacterial communities associated with AMF spores was higher in cultures on H. pilosella than in those on P. lanceolata. This was obvious by optical observation (Fig. 1) and was confirmed by correspondence analysis (Fig. 2). The distances among the samples of two different host plants within one AMF species were shorter than the distances among the samples of the two AMF strains with one plant species, indicating that the bacterial community was structured mainly by the AMF species (Fig. 2). This observation is supported by variation partitioning analysis, which revealed that the host plant explained 12.1% (P = 0.001) of the variation of the bacterial DGGE patterns, that the AMF species explained 21.6% (P = 0.001) of the variation, and that there was no cross-variation (P = 0.12). A strong proportion of bands were common to all the culture conditions (Fig. 3), showing that many bacterial populations were always associated with AMF spores whatever the fungal species and
the host plant were. In addition, many of these common bands had a high relative intensity, indicating that they were probably the most dominant populations on the AMF spores. To determine their affiliations, 11 bands in common (bands 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, and 15) and four bands belonging to particular culture conditions (bands 1, 2, 10, and 11) were excised, cloned, and sequenced. Because of the higher homogeneity of the replicates, only DGGE bands obtained from *H. pilosella* cultures were selected. Band 1_AJ864379 was present only with *G. geosporum* and was affiliated with the phylum *Fibrobacteres* (Fig. 4A), band 2_AJ864380 was present only with *H. pilosella* and was related to the genus *Desulfovibrio* (Fig. 4D), band 10_AJ864388 was present only in *G. constrictum* and *H. pilosella* cultures and was affiliated with the phylum *Fibrobacteres* (Fig. 4A). Band 11_AJ864389 was present only with *G. constrictum* and was affiliated with the genus *Pseudomonas* (Fig. 4E). Three bands were present under all of the culture conditions but with a relative abundance much higher in the case of *G. geosporum*: band 6_AJ864384 was related to the genus *Lysobacter* (Fig. 4E), band 7_AJ864385 was related to the genus *Flexibacter* (Fig. 4B), and band 9_AJ864387 was related to the genus *Chondromyces* (Fig. 4D). Finally, eight bands were found under all culture conditions and with similar relative abundances: bands 3_AJ864381, 8_AJ864386, and 13_AJ864391 were related to the genus *Flexibacter* (Fig. 4B); bands 5_AJ864383 and 12_AJ864390 were related to the genus *Cellvibrio* (Fig. 4E); band 4_AJ864382 was related to the genus *Burkholderia* (Fig. 4C); band 14_AJ864392 was related to the genus *Rhizohyphomusa* (Fig. 4E); and band 15_AJ864393 was related to *Cyanobacteria* (data not shown). Interestingly, most of the genera identified are bacteria that can hydrolyze biopolymers such as proteins, cellulose, and chitin (15, 24, 27, 28, 29). When the mean values of the relative intensities of all the band sequences related to these biopolymer-degrading genera are added up, they represent 60% of the overall intensity in *G. geosporum/P. lanceolata*, 84% in *G. geosporum/H. pilosella*.
pilosella, 53% in G. constrictum/P. lanceolata, and 73% in G. constrictum/H. pilosella cultures. Therefore, these polymer-degrading bacteria probably represent the main populations contributing to the bacterial community associated with AMF spores.

Prior to being prepared for microscopy, G. geosporum spores were divided into the following three consecutive maturity stages discernible under the dissecting microscope: the youngest spores, which were light yellow-brown without or containing a few dark patches; medium-brown spores with many patches; and dark-orange-brown spores with many patches or one giant patch. SEM observations revealed that the thin outer hyaline wall layer of G. geosporum spores was gradually degraded and replaced by mucilaginous products (Fig. 5D and E). The rough surface composed of the degraded and mucilaginous outer hyaline layer was present to different extents depending on the spore maturity stage. Out of 38 light-colored spores observed, only 6 had a smooth surface, 18 were covered with roughness of up to half of the visible surface by SEM, and the surfaces of 14 were entirely rough (Fig. 5A). Out of 24 medium-brown spores, 16 were virtually smooth, 8 were covered with a rough material on 50% of the surface, and 8 were entirely rough (Fig. 5B). Finally, out of 17 dark-colored spores examined, 15 were entirely smooth and only 2 were slightly rough (Fig. 5C). Bacterial cells of different sizes were present either in the sloughing hyaline layer or on the surface of the second, laminated wall layer (Fig. 5F). Decaying material complicated the observation of bacterial cells because they appeared to be covered with their own mucilage. On the smooth laminated surfaces, bacterial filaments were observed covered with mucilage products (Fig. 5G). The spore surface also contained many holes, possibly corresponding to lysis zones (Fig. 5F and H). Finally, many coccus-shaped cells were also present (Fig. 5H and I).

**DISCUSSION**

The bacterial community associated with the *Glomus* spores was more influenced by the AMF identity (G. geosporum or G. constrictum) than by that of the host plant (H. pilosella or P. lanceolata). Despite the impact of the root on its surrounding environment and consequently on the microbial community, the plant did not predominantly affect the spore-associated bacterial community structure. Moreover, there was good homogeneity within replicates. The AMF spores thus seem to provide a microhabitat with particular conditions for the development of specific bacterial populations. The difference in composition of the spore walls or of exudates of these two *Glomus* species may have played a major role in the selection of bacterial populations living on the spore. In addition, the two *Glomus* species were isolated from the same site (35) and subcultured under the same conditions. The subculturing process may have enriched spore-associated bacterial populations adapted either to *G. geosporum* or to *G. constrictum*, which could have increased the discrepancies between the spore-associated bacterial community structures of the two fungi at the time of analysis.

Roughly one-third of the DGGE bands, among which were some exhibiting the highest relative intensities, were found in the profiles of cultures from all conditions. As a whole, these bands represented more than 50% of the relative intensity of the entire profiles. They comprised sequences affiliated mainly with genera with hydrolytic representatives (*Cellvibrio, Chondromyces, Flexibacter, Lysobacter, and Pseudomonas*). These biopolymer-degrading bacteria are possibly feeding on the outer hyaline spore layer that is present in both species and consists mainly of chitin, a straight-chain polymer of N-acetylglucosamine (32). Filippi et al. (9) actually demonstrated that many bacteria were attached to the hyaline wall layer of *Glomo-
**FIG. 5.** SEM images of the surface of *G. geosporum* spores. (A) Young, light yellow-brown spore with its sloughed and eroded outer hyaline layer covering the whole surface; (B) older, medium-brown spore with a residual outer hyaline layer; (C) old, dark orange-brown spore that has lost almost all its outer hyaline layer; (D) outer hyaline layer starting to “peel off” and being replaced by mucilaginous products (arrow); (E) mucilaginous outer hyaline layer; (F) bacterial cells of various shapes adhering to the surface of the laminated layer, with holes possibly corresponding to lysis zones in the spore wall; (G) filamentous bacterial cells adhering to the laminated layer covered with mucilaginous products; (H) coccus-shaped bacteria in division state on the spore surface; (I) chain of coccus-shaped bacteria covered with mucilaginous products. Bars (in micrometers): A and B, 50; C, 100; D, E, F, and G, 10; H and I, 1.
spores germinated in vitro only in the presence of microorganisms, including Streptomyces orientalis. Ames and coworkers (2) found that out of 190 spores examined, 100 were colonized by one or more chitin-decomposing microorganisms; 82% were colonized by actinomycetes, 17% by bacteria, and 1% by fungi. Carpenter-Boggs et al. (7) demonstrated a positive correlation between higher germination rate and the amount of production of geosmin, CO₂, and 2-methylisoborneol by the actinomycetes. In our study, none of the DGGE bands sequenced were affiliated with actinobacteria. As not all the discrete bands mycetes. In our study, none of the DGGE bands sequenced were affiliated with actinobacteria. As not all the discrete bands sequenced were affiliated with actinobacteria. As not all the discrete bands sequenced were affiliated with actinobacteria.

Several bands sequenced were affiliated with genera capable of cellulolytic activity. The presence of cellulolytic bacteria on the spore surface indicates that microorganisms attached to the spores may also degrade plant material around them (e.g., cellulose from sloughed off cortical root cells). Gryndler et al. (13) have reported that an amendment of cellulose, if incubated in the soil for a long time, increased the number of bacteria and saprophytic fungi in the soil and also stimulated AMF growth. They suggested that this AMF stimulation could result from an AMF uptake of nutrients released from the decomposing saprophytic microflora. Root exudates also provide the microorganisms with readily assimilable organic substrates (17) and thus stimulate the growth of the biopolymer-degrading populations that would in turn accelerate the decay of the outer spore walls. Indeed, the outer hyaline layers, which are generally the first component of the spore wall synthesized in juvenile spores, are rarely present on mature spores in the soil (14). The presence of active biopolymer-degrading bacterial populations on the spore surface could support also spore germination by releasing nutrients or degrading toxic compounds that inhibit germination. Thus, the process of matura-

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### References


