ORIGINAL ARTICLE

Isolation and analysis of bacteria associated with spores of *Gigaspora margarita*

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Abstract

Aims: The aim of this work was to observe bacteria associated with the spores of *Gigaspora margarita*, an arbuscular mycorrhizal fungus (AMF).

Methods and Results: First, a direct analysis of DNA from sterilized spores indicated the bacteria belonging to the genus *Janthinobacterium*. In the second assay, two bacterial strains were isolated by osmosis from protoplasts, which were derived from spores by using two particular enzymes: lysing enzymes and yatalase. After isolation, cultivation and identification by their DNA as performed in the first experiment, the species with the closest relation were *Janthinobacterium lividum* (KCIGM01) and *Paenibacillus polymyxa* (KCIGM04) isolated with lysing enzymes and yatalase respectively. Morphologically, *J. lividum* was Gram negative and oval, while *P. polymyxa* was also oval, but Gram positive. Both strains had antagonistic effects to the pathogenic fungi *Rosellimia necatrix*, *Pythium ultimum*, *Fusarium oxysporum* and *Rhizoctonia solani*. In particular, *J. lividum* was much stronger in this role. However, in phosphorus (P) solubilization *P. polymyxa* functioned better than *J. lividum*.

Conclusions: This experiment had revealed two new bacteria species (*P. polymyxa* and *J. lividum*), associated with AMF spores, which functioned to suppress diseases and to solubilize P.

Significance and Impact of the Study: AMF spores could be a useful source for bacterial antagonists to soil-borne diseases and P solubilization.

Introduction

Arbuscular-mycorrhizal fungi (AMF) live as obligate symbiosis with the roots of about 80% of land plants, and this symbiotic relationship exists in most natural and agricultural ecosystems. Their importance for plant health, nutrient cycling and soil quality are well reported (Ishii *et al.* 1997).

There are many publications about specific bacteria that promote interactions between AMF and plants. This may serve as a third partner in this symbiosis (Azcón-Aguilar *et al.* 1998; Rillig 2004). The cytoplasm of AMF spores contains some intracellular structures similar to bacteria called bacterium-like organisms (BLO), frequently located in the vacuoles (Bonfante *et al.* 1994; Cruz 2004). Morphological observations with electron and optical microscopes, combined with molecular analyses suggested that those BLO were true bacteria, which live in close association with AMF. The *Gigaspora margarita* species harbours a homogeneous population of endobacteria in its cytoplasm throughout its life cycle, and the high amounts of bacterial cells during successive generations suggest that those bacteria are part of the fungal system (Minerdi *et al.* 2001). The amplification of bacterial 16S RNA gene from spores with direct sequencing indicated a close relationship of these bacteria with genus *Burkholderia*, from the family βproteobacteria (Banciotto *et al.* 1996). This genus contains a *vacB* gene, which might be involved in the symbiotic interaction with *G. margarita* (Ruiz-Lozano and Bonfante 2000). Some bacterial strains isolated from spore's surface functioned on phosphorus (P) solubilization (Viveganandan and

Jauhri 2000), diseases suppression (Lioussanne *et al.* 2006), and as ethylene producers, which contributed indirectly to AMF hyphal growth (Horii and Ishii 2006). Furthermore, a bacterium (*Paenibacillus* sp. strain B2), isolated from the mycorrhizosphere of *Sorghum bicolor* inoculated with *Glomus mosseae* had antagonistic effect on soil borne fungal pathogens and could stimulate mycorrhization (Budi *et al.* 1999).

Although the occurrence of endobacteria in spores, which increase the complexity of AMF (Bonfante 2003), is well demonstrated, their physiological roles in fungal fitness and in mycorrhizal symbiosis are still unknown. Moreover, their cultivation *in vitro* has been set aside because of the difficulty to grow in cell-free media (Minerdi *et al.* 2002). Except the bacterium strain 'Candidatus *Glomerbacter gigasporarum*', obtained from *G. margarita* spores (Jargeat *et al.* 2004), could be kept alive for several weeks. At least these authors succeeded in isolating enough of this bacterium to reveal some information on their morphology, physiology and genome structures even though there were difficulties because of low fungal biomass, and bacterial density in AMF spores. In another research, this bacterium could vertically be transmitted through fungal spore generations, and its absence affected presymbiotic fungal growth (Lumini *et al.* 2007). Bacteria of genus *Paenibacillus* were also shown to live intracellularly in ectomycorrhizal fungus (Bertaux *et al.* 2003), promoting the fungal growth in this symbiosis.

The difficulty of isolation and cultivation of bacteria taken from spores in cell-free media might be because of their location in vacuoles. Perhaps the protoplasts could remove the bacteria from inside spores without causing damage. Some bacteria, after extraction from spores, require specific conditions for acclimatization to the media, such as C source, partial O_2 or pH (Jargeat *et al.* 2004). In most cases, they are obligate symbiont, indicating their strictly physiological dependence upon the spores. This study investigated the existence of some species besides those already identified with a hope to evaluate a method to isolate these bacteria osmotically, with posterior cultivation. The isolation by osmosis synergistically through a strong sterilization could lead to identify new bacterial species, probably located inside the spores.

Materials and methods

Experiment 1 – Identification of associated bacteria from *G. margarita* spores

About 100 spores of *G. margarita*, obtained from a commercial inoculum at Central Glass Co., Tokyo, Japan, previously multiplied in bahiagrass culture and preserved in peatmos substrate under dark conditions at 4°C, were taken by wet sieving, and by the criteria of colour the clearest spores were selected. These spores were washed and surface sterilized for 30 min with a 14% of solution containing chloramines-T (7000 ppm), streptomycin (56 ppm), chloramphenicol (20 ppm) with a few drops of Tween 80. They were then washed with sterilized distilled water (DW) seven times. In a previous experiment, 15 min sterilization on spores did not affect the germination, but 20 min was enough to kill the entire microorganisms surrounding the spores (Horii and Ishii 2006). Therefore, we decided to insert the spores into antibiotic solution for 30 min to guarantee complete surface sterilization.

After the sterilization process, crude DNA extraction from spores was carried out by inserting them into polymerase chain reaction (PCR) tubes and homogenization by alkali method. To proceed with this, 2 μl of NaOH (0·25 mol l^{-1}) was added into the tubes with crushed spores and boiled for 1 min. Then the spores were treated with 1 μl of Tris–HCl (0·5 mol l^{-1}) adjusted to pH 8·0 and 2 μl of HCl (0·25 mol l^{-1}) boiled again for 2 min. Then they were centrifuged at 18 659 *g* for 3 min and the supernatant was recovered. To avoid bacterial contamination, extreme care was taken during the sterilization and alkali treatment method.

Bacterial 16S rDNA was amplified in 50 μl volume containing 1 μl of crude DNA preparation, 2·5 μl of each bacterial primer, 27f/1492r, KOD Dash (2·5 U μl^{-1}, Toyobo, Tokyo, Japan) or r*Taq* (2·5 U μl^{-1}, Toyobo) as *Taq* polymerase, 5 μl of dNTPs (2 mmol l^{-1} dATP, dGTP, dCTP, dTTP each, Toyobo) and 5 μl of 10× reaction buffer (Toyobo). Sterilized DW was added to complete the volume. The oligonucleotide primers for 16S rDNA partial sequence specific PCR, 1492r: 5′-GGCTACCTTGTTACGACTT-3′ and 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ (Hiraishi 1992), were made by Proligo (Tokyo, Japan). The PCR cycling conditions for KOD Dash were one cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 2 s and 74°C and 30 s and final extension step at 20°C for 3 min. For r*Taq*, the PCR was carried out as follows: one cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, and 74°C for 45 s, and the final extension step was at 72°C for 3 min.

The PCR products were purified using Gene clean Kit (Q-BIOgene, Solom, OH, USA) according to the manufacturer's instructions, and the PCR purified products were used in sequencing reactions with the 27f primer using a BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems). The sequencing was performed using a Genetic Analyzer 310 (Applied Biosystems, Foster City, CA, USA), which searched the nucleotide sequence database using BLAST programs. After that, the sequence

was scanned for its similarity with those in the data bank and manually aligned with groups representing the bacterial lineages. Phylogenetic analysis was conducted with the Bioedit 7.0.0 and Tree View 1.6.6 programs on sequence alignments, which included and excluded the 16S DNA variable regions. The phylogenetic relationship was carried out by neighbour-joining analyses, and Kimura's parameter genetic distances. This was combined with bootstrap analyses from 1000 replicates.

Experiment 2 – Isolation, identification and function of bacteria from *G. margarita* spores

First, two portions of 100 spores were selected and sterilized as described in the experiment 1. After sterilization, a sample of 10 spores were put on two kinds of growth media, agar 1·5%, and peptone media for 4 days to verify the efficiency of sterilization by observing the hyphal growth and the presence of micro-organisms surrounding the spores. The remaining portion was then submitted to sequential osmotic solutions for 17 days to isolate the bacteria. First, protoplasts were prepared from spores by using a modification of another procedure (Yelton *et al.* 1984). A basic solution (1·2 mol l^{-1} $MgSO_4$/10 mmol l^{-1} Na_2HPO_4, pH 5·8) was prepared to be used in two types of high osmotic solution (HOS): one containing lysing enzymes and other Yatalase at concentration of 5 mg ml^{-1}. Then these HOS were filtrated through a 0·2-μm mesh screen. This homogenization composed two HOS, one with lysing enzymes and other with yatalase. The spores were then inserted into the tubes containing 1 ml of HOS, kept in ice for 5 min, and shaken at 80 rev min^{-1} at 30°C for 3 days. After that, 500 μl of this HOS was carefully replaced by sterilized DW, making a low osmotic solution (LOS), and kept at room temperature for 3 h. When this stage was finished, the full amount of LOS was removed, completely replaced with DW and kept in the same condition for 2 weeks. A separated flash containing the same protoplasts without the spores was prepared as control.

After completing the process of osmotic removal, a sample of 50 μl DW from the tubes, including the control ones, was put on Petri dishes containing 10 ml of peptone media to grow the bacteria for a week. The Petri dishes containing the DW from the control flash did not grow any bacteria. The whole system, including the bacteria removal, was conducted aseptically to avoid contamination.

The bacteria from the Petri dishes were transferred into tubes with liquid media composed of polypepton and bacto™ yeast extract at 5 g and 1 g l^{-1}, respectively, and shaken aseptically for 24 h at 26°C. A sample of 200 μl of the bacteria grown in liquid media was inserted in tubes and centrifuged at 11 519 ***g*** for 1 min. The supernatant was discarded and the remaining bottom phase was submitted to DNA extraction by using Isoplant DNA extraction kit (Nippon Gene, Tokyo, Japan). This solution was used as the template of PCR, followed by sequencing as explained in experiment 1. The maximum similarity of sequences to identify the species was chosen by the criteria of higher identity (%) and lower *E*-value.

To evaluate the morphology of the bacteria, a Gram test was carried out (Akita-University 2004). P solubilization was determined (according to Gaind and Gaur 1991) with some modifications where the soluble P was analysed by the ascorbic acid method as described by (Alam *et al.* 2002). To evaluate the disease suppression; four pathogenic fungi: *Roselinea necatrix, Pythium ultimum, Fusarium oxysporum* and *Rhizoctonia solani*, were incubated separately in the centre of Petri dishes containing potato dextrose agar. Two discs with the bacteria were put on the periphery of these dishes. After 4 days of incubation at 27°C, the apparent area that surrounded the bacterial disc compared with the control (no bacteria) was used to estimate the degree of antagonism based on the criteria of number scale. The morphological features were observed through a light microscope using a thin layer of the bacteria with a drop of distilled water dried in a clean bench.

Results

Phylogenetical analyses of bacteria

Through the database search for sequence similarity and phylogenetic analyses of 16S rDNA, we found basically that the main family was Oxalobacteraceae, which belongs to Burkholderiales order and the class of βproteobacteria. This family harbours two genera, *Janthinobacterium* and *Massilia*. The isolated bacterium belongs to the genus *Janthinobacterium*, but the bootstrap values were low (Fig. 1). The tree (neighbour-joining method; Kimura's correction) was obtained after the complete exclusion of deletions and/or variable regions.

Analyses and function of the bacteria isolated and cultured

After sterilization no hyphal growth was observed in the Petri dishes with agar and there was no micro-organism growth on the area that surrounded the spores in the peptone media, confirming the efficiency of the sterilization process.

Using the ribosomal DNA sequence-based molecular biological methods, two bacterial strains were identified (Table 1). The identical species (Gene Bank accession no., identities, E-value) are: KCIGM01, *Janthinobacterium*

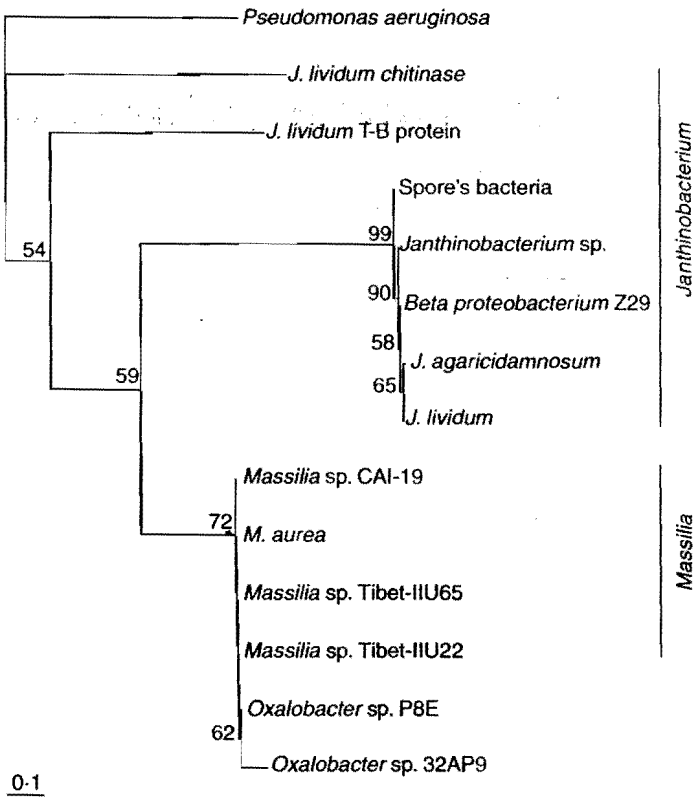

Figure 1 Phylogenetic placement of spore bacteria from *Gigaspora margarita* spores. Neighbour-joining analysis was performed with clustalx using Kimura's distance method combined with bootstrap analysis from 1000 replicates (bootstrap values <50% not shown). The tree was rooted with *Pseudomonas aeruginosa*.

Table 1 Identification of two kinds of bacteria located inside of *Gigaspora margarita* spores isolated by osmosis

Items	KCIGM01	KCIGM04
Isolation method	Osmosis with lysing enzymes	Osmosis with yatalase
Specie on database	*Janthinobacterium lividum*	*Paenibacillus polymyxa*
Gene bank no.	AY788973	AY302439
Identity (%)	99	99
E-value	0·0	e-120

Table 2 Morphological characteristics and functions of two kinds of bacteria located inside of *Gigaspora margarita* spores isolated by osmosis

Items	KCIGM01 (*Janthinobacterium lividum*)	KCIGM04 (*Paenibacillus polymyxa*)
Gram test	−	+
Outshape	Oval	Oval
*,†Pathogens' inhibition		
Roselinea necatrix	2·5 ± 0·2	0·0 ± 0·0
Pythium ultimum	1·0 ± 0·1	0·3 ± 0·1
Fusarium oxysporum	2·1 ± 0·3	0·8 ± 0·2
Rhizoctonia solani	2·4 ± 0·2	0·2 ± 0·1
*Phosphorus solubilization (%)	10·6 ± 0·7	15·0 ± 1·2

*Mean ± standard error, $n = 4$.
†Inhibition level scale: 0-no; 1-slightly; 2-moderate; 3-strong.

lividum (AY788973, 99%, e-0·0); and KCIGM04, *Paenibacillus polymyxa* (AY302439, 99%, e-120) and were extracted by using lysing enzymes and yatalase respectively.

Both bacteria had similar morphological characteristics. The acquired species on the basis of the molecular biological identification were *J. lividum*, which had an oval shape measuring between 2–4 μm length and 1–2 μm of diameter, and the *P. polymyxa* which too had an oval shape with 3–6 μm length and 1–2 μm of diameter. The gram staining test indicated that *J. lividum* and *P. polymyxa* were negative and positive respectively (Table 2).

Concerning the ability of bacteria to suppress the growth of pathogens, *P. polymyxa* had an antagonism scale ranging from 0 to 0·8, which is considered slight, except for the *R. necatrix* where this bacterium did not show any effect. However, *J. lividum* had moderate to strong antagonism to fungal pathogens, especially against *R. necatrix* where its growth in some Petri dishes was strongly suppressed by the presence of this bacterial strain, compared with the Petri dishes without bacteria. Both strains also functioned well to P solubilization, but in this function, *P. polymyxa* was able to solubilize a

higher amount of P than *J. lividum* as demonstrated by the values obtained (Table 2).

Discussion

This research revealed that *G. margarita* spores might harbour another bacterium genus (*Janthinobacterium*) in addition to those already reported. But, the relationship between this genus and AMF spore is not yet defined as symbiotic. The taxonomy shown in the tree relates two genera from the Oxalobacteraceae family, *Massilia* and *Janthinobacterium*, but the tree obtained in this work demonstrates that these bacteria probably belong to the *Janthinobacterium* group, as they are located in a better branch nested in this genus and are far from the *Massilia*. The identification of this micro-organism as member of genus *Janthinobacterium* is also supported by the bootstrap analysis. Although the genus *Glomaribacter* (Jargeat *et al.* 2004) has been well detected in *G. margarita* spores, in our material, this genus could not be observed. Perhaps, different bacteria associated with AMF spores reflect differences in microbial community of soils where spores were collected.

This eubacterial taxon belongs to the family of βproteobacteria (Garrity *et al.* 2003), and mostly consists of two species, *J. lividum* and *Janthinobacterium* sp. This genus can be found in soil and water (Moss and Ryall 1981) and exhibits a high degree of genetic variations (Saeger and Hale 1993). This bacterium produces acid from the fermentation of carbohydrate xylose (De-Ley *et al.* 1978; Sneath 1984), and is known to be chitinolytic because of its property to degrade fungal cell wall (de-Boer *et al.* 2004). Soil bacteria may be genetically more diverse than those with specific hosts. Thus, the strains found in AMF spores might have less genetic variations than those isolated from soil or aquatic environments (McArthur *et al.* 1988). In our first experiment, the spores were sterilized with antibiotics, which sometimes are insufficient to prevent the amplification of bacteria harboured in fungal cell wall unable to grow in media (Hosny *et al.* 1999). Therefore, the second experiment was carried out to confirm the bacterial strain found in the first one.

In the methods discussed in this paper, it was essential that we used two different enzymes, which allowed for the isolation of two bacterial strains. A possible explanation would be that the protoplast was able to remove the bacteria from spores without affecting their original characteristics, and allow them to be cultivated in cell-free media. Many studies have demonstrated the presence of endosymbiont bacteria in AMF spores (Bonfante 2003), including their phylogenetic analyses, but only a few of them have been identified (Minerdi *et al.* 2002; Banciotto *et al.* 2003).

The bacteria reproduced in media and identified with *J. lividum* had an oval shape and was Gram negative with a high degree of sequence similarity to members of the genus *Janthinobacterium*. The morphological features of the bacteria such as size and format confirmed the information provided by other authors, where the *J. lividum* was Gram negative ranging from 0·8 to 15 μm in diameter and from 1·8 to 6 μm in length (Euzéby 2004). Also the *P. polymyxa* had an oval spore shape, and was gram variable (negative or positive) (Shida *et al.* 1997).

In this study, both *P. polymyxa* and *J. lividum* functioned very well to solubilize P where *P. polymyxa* was more efficient. In terms of biocontrol, the *J. lividum* was moderate to strong especially against *R. necatrix* whose growth was prolonged, but *P. polymyxa* was only slightly active in this function, contrary to the other results obtained with this same strain (*P. polymyxa* KY1091) isolated from the rhizosphere of bahiagrass (*Paspalum notatum* Flügge) (Ishii *et al.* 2006). This suggests that the strain obtained in the current work may be different to those from Ishii *et al.* (2006). Another explanation could be that the bacterium's ability to suppress the pathogen growth may change according to the environment. However, we still hypothesize that the biocontrol activity occurring in soils with AMF is linked to these associated bacteria. Although the functional significance of a bacterium found in spores remains unknown, its presence in fungi may be mandatory, introducing a new complexity in mycorrhizal association (Margulis and Fester 1991). This information also suggests that the role of species found in AMF spores might be different from those found in soil and water. The genera *Paenibacillus* has been associated with AMF, especially contribution *in vitro* (Hildebrandt *et al.* 2002). These authors demonstrated that the *P. validus*, a bacterium isolated from the surface of AMF spores, was able to support the growth of the fungus *Glomus intraradices* and to form new spores *in vitro*. More specifically, *P. polymyxa* and *P. macerans*, have been associated with nitrogen fixation (Coelho *et al.* 2002), P solubilization (Viveganandan and Jauhri 2000) and the suppression of plant pathogens (Budi *et al.* 1999; Lioussanne *et al.* 2006; Yasuda *et al.* 2006). The role of *P. polymyxa* and *J. lividum* in AMF spores remains unclear, but theoretically, the association with bacteria is obligatory for AMF survival (Banciotto *et al.* 1996), and their activities in the spores might or might not affect the AMF relation with plants.

With regard to the location of these bacteria, some evidences could show that perhaps they were originated from the inside of *G. margarita* spores: (i) after 30 min of sterilization, no bacteria was able to grow on spore surface, even though 20 min was enough to sterilize and strongly inhibit spore germination (Horii and Ishii 2006); (ii) the DW from the control tubes (without spores)

during the osmotic process did not grow any microorganism after place on growth media; (iii) the morphological features are very similar to previous results shown in some pictures taken from the inside of *G. margarita* spores by transmission electronic microscope (Bonfante *et al.* 1994; Cruz 2004). These bacterial species are usually in soil or water (*J. lividum*). One possibility could be that they might have penetrated into spores through holes on spore's walls by degradation of cells, such as *J. lividum* in this current generation or before. The genus *Paenibacillus*, often found on spores surface (Lioussanne *et al.* 2006), is also frequent in soils. Furthermore, the *Burkholderia* located inside spores (Banciotto *et al.* 1996; Bonfante 2003) was also isolated from bahiagrass (*Paspalum notatum* Flügge) and naginatagaya (*Vulpia myuros* (L.) C. C. Gmel.) rhizospheres (Yasuda *et al.* 2006). Therefore, bacterial living in other environments can also be located inside spores, probably as a way to survive in unfavourable conditions.

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