Detection and Identification of Novel Intracellular Bacteria Hosted in Strains CBS 648.67 and CFCC 80795 of Biocontrol Fungi Metarhizium

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“Endosymbiosis” is a cohesive form of a symbiotic association. Endobacteria exist in many fungi and play important roles in fungal host biology. Metarhizium spp. are important entomopathogenic fungi for insect pest control. In the present study, we performed comprehensive analyses of strains of Metarhizium bibionidarum and M. anisopliae using PCR, phylogenetics, and fluorescent electron microscopy to identify endobacteria within hyphae and conidia. The results of the phylogenetic analysis based on 16S RNA gene sequences indicated that these endobacteria were the most closely related to Pelomonas puraqua and affiliated with Betaproteobacteria. Ultrastructural observations indicated that endobacteria were coccoid and less than 500 nm in diameter. The basic characteristics of endobacteria in M. bibionidarum and M. anisopliae were elucidated, and biological questions were raised regarding their biological functions in the Metarhizium hosts.

Key words: endosymbiosis, endobacteria, Metarhizium bibionidarum, Metarhizium anisopliae

In natural ecosystems, symbiosis is an important association between animals, plants, fungi, and prokaryotes (Frey-Klett et al., 2007; Kikuchi, 2009; Giordano et al., 2013). Symbionts often play critical roles in development, physiology, behavior, defense, and a number of other traits in a host (Selosse et al., 2004; Leung and Poulin, 2008; McFall-Ngai et al., 2013; Gerardo and Parker, 2014). Symbiotic relationships in which both species in the association benefit are mutualistic (Das and Varma, 2009). Mutualistic relationships between bacteria and hosts (animals, plants, and fungi) are very common (Fraune and Bosch, 2010; Bonfante and Desiro, 2017; Zipfel and Oldroyd, 2017). In mutualism, hosts often provide bacteria with a living space and the nutrients needed for growth (Anca et al., 2009; Schüller, 2012; Bonfante et al., 2019), while bacteria play important roles in host biology, including aspects of host reproduction (Partida-Martínez et al., 2007; Mondo et al., 2017), growth (Guo et al., 2017; Shaffer et al., 2017; Desiro et al., 2018), energy dynamics (Vannini et al., 2016; Salvioli et al., 2017), primary metabolism (Vannini et al., 2016; Li et al., 2017; Uehling et al., 2017), and secondary metabolism (Rohm et al., 2010; Hoffman et al., 2013; Deveau et al., 2018). In addition to living on the body surface or within the tissues of the host in the extracellular space, bacteria may also inhabit the cytoplasm, forming the most intimate interaction, called “endosymbiosis”. These bacteria are termed cytoplasmic endobacteria (Bonfante et al., 2019).

Endosymbiosis between bacteria and plants/animals has been extensively examined for many years (Haney and Ausubel, 2015; Santos et al., 2018; Skiai et al., 2019); however, the study of bacterial-fungal interactions has only emerged in recent years (Bonfante and Desiro, 2017). Several fungal lineages possess endobacteria, including Mucoromycota, Glomeromycota, Ascomycota, and Basidiomycota (Hoffman and Arnold, 2010; Deveau et al., 2018; Liu et al., 2019). Known endobacteria include Alphaproteobacteria, Betaproteobacteria, Firmicutes, Actinobacteria, and Gammaproteobacteria, and the functions of these endobacteria in their hosts have been preliminarily investigated and speculated (Rohm et al., 2010; Liu et al., 2019).

Recent studies on fungal endobacteria have mainly focused on the endobacteria of fungi with plant hosts (Bonfante et al., 2019; Liu et al., 2019), while limited information is currently available on the endobacteria of entomopathogenic fungi. Metarhizium spp. are natural enemies of a wide range of insects and arachnids, and, thus, they are being used as environmentally friendly alternatives to chemical insecticides (Gao et al., 2011). Metarhizium spp. are one of the most widely used fungi and mycoinsecticides worldwide (Zimmermann, 2007), with Metarhizium acridum being used to manage and prevent infestations by the superfamily Acridoidea (Gao et al., 2011). In addition, M. anisopliae is effective in the control of malaria-vectoring mosquitoes (Culicidae, Diptera) as well as scarabs and aphids (Scholte et al., 2005; Kanzok and Jacobs-Lorena, 2006; Schrank and Vainstein, 2010). The phylogeny (Driver et al., 2000; Bischoff et al., 2009), infection mechanisms, virulence (Schrank and Vainstein, 2010), safety, environmental stability (Zimmermann, 2007), strain improvements (Roberts and Leger, 2004), and other

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aspects of *Metarhizium* spp. have been extensively examined; however, the endobacteria of *Metarhizium* spp. remain unknown.

Through preliminary bacterial sequence amplification and fluorescent nucleic acid staining, we detected endosymbiotic bacteria in *Metarhizium* spp. Endobacteria may be important to the biology and ecology of *Metarhizium* spp. To obtain a more detailed understanding of the presence and spatial distribution of endobacteria in *Metarhizium* spp., we investigated the domestic and foreign strains, CBS 648.67 (Nishi et al., 2017) and CFCC 80795, respectively. CBS 648.67 and CFCC 80795 belong to the two most common species of *Metarhizium* spp., *M. bibionidarum* (a member of the *M. flavoviride* species complex) and *M. anisopliae*, respectively. These strains of the genus are also commonly used in *Metarhizium* insecticides (Milner, 2000; Nong et al., 2015; Brunner-Mendoza et al., 2018; Mascarín et al., 2019). Previous pathogenicity experiments against *Monochamus alternatus* adults revealed the good insecticidal efficacies of these two strains (Y. Ying et al., unpublished). In the present study, the ultrastructural morphology and localization of endobacteria in hyphae and conidia were confirmed using a combination of fluorescence *in situ* hybridization (FISH), transmission electron microscopy (TEM), and scanning electron microscopy (SEM). The phylogeny of endosymbions was revealed by sequencing and phylogenetic analyses. The results obtained herein revealed the presence of endobacteria in *Metarhizium* spp., and provide a new perspective for the complex interactions between *Metarhizium* spp., endobacteria, and host insects.

**Materials and Methods**

**Fungal strain**

The biological control fungal strain CBS 648.67 (*M. bibionidarum*) was purchased fromCentraalbureau voor Schimmelmicrocollections (CBS), and strain CFCC 80795 (*M. anisopliae*) was provided by the China Forestry Culture Collection Center.

**Fungal culture conditions**

The mycelia or conidia of fungal cultures were used to inoculate solid potato dextrose agar (PDA; Becton, Dickinson and Co.) medium or liquid potato sucrose (LPS; 200 g fresh potato and 20 g sucrose [Sinopharm Chemical Reagent]) medium at 25°C.

**Isolation and culture of endobacteria**

The mycelia of 7-day-old CBS 648.67 and CFCC 80795 grown in liquid-submerged cultures were filtered and cryo-ground (0–4°C) using RETSCH’s Mixer Mill MM 400. After grinding, products were mixed with 0.25 M sucrose solution and centrifuged at different rotational speeds to obtain isolated bacteria. Supernatants were collected and filtered through cellulose nitrate (Cellulose Nitrate [Mixed Cellulose Ester] Membrane Filters, 5 μm, Sartorius) to obtain suspensions containing endobacteria. They were then centrifuged at 13,000×g at 4°C for 20 min. The supernatants were gently removed to avoid losing endobacteria, which were resuspended in 0.25 M sucrose solution. Suspensions were plated onto agar plates and incubated at 25°C.

Each bacterial suspension was spread onto two media (200 μL of suspensions per plate): LB agar (L−1: 10 g tryptone [Becton, Dickinson and Co.], 10 g sodium chloride [Sinopharm Chemical Reagent], 5 g yeast extract [Becton, Dickinson and Co.], and 1.5% [w/v] agar [Sinopharm Chemical Reagent]) and R2A agar (Becton, Dickinson and Co.), and then incubated at 25°C for 2 weeks. R2A agar is suitable for *Pelomonas* growth according to Gomila et al. (2007), and LB agar is commonly used as a bacterial medium. Therefore, these two media were selected for the cultivation of endobacteria. Ten plates were used to observe the growth of endobacteria.

**DNA extraction, amplification, and clone library analysis**

Fungal hyphae were filtered under sterile conditions and ground to a fine powder in liquid nitrogen. DNA was isolated using the TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa Bio) in accordance with the instruction manual in a sterile environment. Endobacterial 16S rRNA genes and mreB cytoskeletal protein-encoding genes were amplified using the universal bacterial primer pairs 27F (5′-GAGAGTTTGTATCCGCTTCAG-3′) / 1492r (5′-TACGGYTACCTTGTAGACTT-3′) (Weisburg et al., 1991) and mreB-1 (5′-GATGAATCCATCCATGCAATCTG-3′)/mreB-2 (5′-CCACCTCGAGTTACCAAATTCCTTGAAG-3′) (Gaballah et al., 2011), and ten pairs of primers (Table S1) were designed using Primer3Plus (Untergasser et al., 2007) based on the approximately 1,043-bp mreB gene of *Pelomonas* spp. in NCBI. The PCR program consisted of 94°C for the initial 2 min, 30 cycles at 95°C with 1 min of denaturing, at 52°C with 90 s of annealing, and at 68°C with 2 min of extension, followed by a final extension for 10 min. The primers 19F (5′GCWTTYAXYGIAARGGIGG-3′) and 407R (5′-AIICCCRCCRCACIAICACRTCT-3′) were used to amplify the approximately 388-bp nifH gene encoding the dinitrogen reductase iron protein. Reaction conditions were an initial step at 94°C for 5 min, 40 cycles at 94°C for 30 s, at 50°C for 1 min, and at 72°C for 30 s, followed by a final step at 72°C for 10 min (Ueda et al., 1995). The primers hoxF1 (5′-GAYCCNRTNACNMGHARGGGGC A-3′) and hoxR1 (5′-ACRTGNRBYSVRANCSCVRDVMANGG RTC-3′) were used for the amplification of the approximately 1,800-bp hydrogenase gene *hoxG* (Kortlücke et al., 1992). The PCR program was used as an initial denaturation step at 94°C for 5 min, 5 cycles at 94°C for 1 min, at 65°C for 1 min, and at 72°C for 105 s, 20 cycles at 94°C for 1 min, at 65°C for 1 min (a decrease of 0.5°C per cycle), and at 72°C for 105 s; 15 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 105 s; and finally an extension at 72°C for 10 min (Gomila et al., 2007).

PCR amplification was accomplished using the Gene Amp PCR System 2400 (PerkinElmer). The 50-μL PCR reaction volume contained 0.25 μL DNA Polymerase LA Taq (TaKaRa Bio.) (5 U μL−1), 5 μL 10× PCR buffer (Mg2+ Plus), 4 μL dNTP mixture (2.5 mM), 1 μL of each primer (20 μM), and 2.5 ng DNA.

PCR products from bacterial amplification were cloned using the pGEM-t Easy Vector System (Promega) and then transformed into DH5α Chemically Competent Escherichia coli (Invitrogen). Ten colonies harboring bacterial fragments were sequenced and analyzed. Colonies were screened for insert lengths. Bacterial clones were sequenced on an ABI 3730xl automated sequencer (Applied Biosystems).

Sequence data were deposited in the NCBI database under the accession numbers MZ686431, OM530146, and MZ822939. MZ686431 and OM530146 were the accession numbers of the 16S rRNA gene sequences of CBS 648.67 and CFCC80795, respectively. MZ822939 was the sequence amplified by the primer pairs mreB-1/mreB-2.

**Phylogenetic analysis**

The 16S rRNA gene sequences of endobacteria in the two strains, sharing >98% identities with the BLAST hits of target sequences from GenBank (30 sequences), were aligned using MUSCLE version 3.8.31 (Edgar, 2004). We also included the closely related genus *Thiomonas*, with *Thiomonas thermosulfata* strain ATCC 51520 serving as the outgroup. JModelTest was used to choose the substitution model for the phylogeny (Guindon and Gascuel, 2003; Darriba et al., 2012).

Phylogenetic analyses were performed using Bayesian and
Maximum-likelihood methods. Bayesian tree sampling was conducted using the MrBayes 3.2.7 program (Ronquist et al., 2012). We employed the Tamura-Nei model of nucleotide evolution, including estimations of invariant sites and the assumption of a discrete gamma distribution (TrN+I+G). The inference consisted of 1,998,000 generations (stopped automatically due to the convergence of all parameters) with sampling every 100 generations. The first 25% of samples were discarded as burn-in (Ronquist et al., 2012). Maximum-likelihood analyses were performed using the program MEGA version 7.0 (Kumar et al., 2016), and maximum-likelihood trees were constructed using the TrN+I+G model and 1,000 bootstraps replicates (Edgar, 2004; Kumar et al., 2016). Tree results were viewed and edited using the program FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

**Localization of endobacteria by fluorescence**

**Fluorescent nucleic acid stain**

The SYTO 9 green-fluorescent nucleic acid stain (Molecular Probes) and microscopic examinations were used to establish whether the small intracellular bodies observed within hyphae were bacteria. In accordance with the manufacturer’s instructions, 1.5 μL SYTO 9 was added per ml fungal suspension. The suspension and dye were mixed thoroughly and incubated at room temperature in the dark for 20 min. Stained hyphae and conidia were viewed using a confocal laser scanning microscope (Leica TCS SP5, Leica). The hyphae and conidia of four cultures of each strain were observed.

**FISH**

One-week-old fungal cultures grown on PDA were harvested and fixed in 50% ethanol with phosphate-buffered saline (pH 7.0) at 4°C for 24 h. Cultures were then washed three times in 1× PBS and treated with Viscozyme® L (Sigma-Aldrich) at 37°C for 2 h and 0.2 M HCl at 25°C for 0.5 h to increase cell wall permeability. Fixed fungal material was dehydrated in an increasing ethanol series (50, 80, and 100% [v/v]) for 3 min each. Endobacteria suspensions (see *Isolation and culture of endobacteria*) were centrifuged at 12,000 g for 5 min. Supernatants were removed and centrifugation was repeated after the addition of distilled water for washing. Distilled water was added to resuspend pellets. FISH was performed as described by Fuchs et al. (2007) and Sharma et al. (2008).

Between 2 and 20 μL of the treated cell suspension (depending on cell density) was spotted onto the wells of “PTFE” Printed Slides (Electron Microscopy Sciences), air-dried, and then dehydrated for 3 min each in 50, 80, and 100% ethanol. Hybridization in 10 μL of the hybridization mix (including 10 μM of each probe) per well was performed at 46°C for 3 h, followed by a stringent washing step at 4°C overnight. After the fixation step, each sample was rinsed three times in 0.1 M PBS buffer (pH 7.0) on ice. Specimens were then dehydrated. The dehydration process included the immersion of specimens in 50% (v/v) ethanol at 4°C for 10 min, 70% (v/v) ethanol for 10 min, 80% (v/v) ethanol for 10 min, 90% (v/v) ethanol for 15 min, and 100% absolute ethyl alcohol (dried with CaCl₂) twice for 20 min. Specimens were then critical-point dried with CO₂ as a transitional fluid and finally sputter coated with gold-palladium using an ion-sputtering device (JFC-1100; JEOL). Each sample was observed and photographed using a scanning electron microscope (S-4800; Hitachi). Four cultures of each strain were observed.

**Results**

**SYTO 9 staining of fungal conidia**

SYTO 9-stained DNA fluoresces bright green under blue excitation. The axenic conidia of *M. anisopliae* were stained with SYTO 9 dye. Many green fluorescent signal spots were observed in the cytoplasm of conidia in addition to fungal nuclei (Fig. 1). These green fluorescent dots indicated endobacteria or other DNA-containing organelles in fungal conidia.

**Isolation and cultivation of endobacteria**

The endobacteria isolated from CBS 648.67 and CFCC 80795 were cultured in two culture media at 25°C for 2 weeks under sterile conditions to investigate their free-living capacities. Endobacterial growth was not observed in any of the media tested.

**Endobacterial identification and phylogenetic tree construction**

Bacterial 16S rRNA gene sequences were amplified by PCR using the universal bacterial primer pair 27f/1492r and sequenced to detect the presence of endosymbionts. Phylogenetic affiliations were then analyzed. PCR experiments revealed that endobacteria were always present in...
the surface-sterilized hyphae and conidia of the two strains (Fig. 2). Database searches using these sequences as queries indicated shared highest identity levels with *Pelomonas* spp. sequences in the NCBI database. Furthermore, a phylogenetic analysis based on 16S rDNA revealed that the endobacterial sequences from the two fungal strains of different geographic origins all clustered in a well-supported clade that was the most closely related to *Pelomonas puraquae* and affiliated with *Betaproteobacteria* (Fig. 2).

Since endobacteria were broadly present inside two different *Metarhizium* strains, their occurrence did not appear to be an accidental or sporadic phenomenon.

Nitrogenase (*nifH*) and hydrogenase (*hoxG*) genes was amplified using the primer pairs 19F/407R and *hox*F1/*hox*R1. Gel images of the PCR products (Fig. S1) generated using the two primer pairs showed multiple bands; however, multi-band clones were not related to the *nifH* or *hoxG* genes of *Pelomonas* spp. Therefore, the *nifH* and *hoxG* genes of the endobacteria associated with *Pelomonas* spp. were absent.

Eleven primer pairs were used for the amplification of the *mreB* gene. A 1,471-bp sequence was obtained using the primer pair *mreB*-1/*mreB*-2. Its length and alignment revealed that it was not the *mreB* gene of *P. puraquae*, but a sequence with an unknown function. The sequence was highly similar to one from *M. brunneum* strain CP058938.1 (query cover 98%, identity 96.14%) according to the BLAST results in the NCBI database. These results also showed that none of the sequences amplified using the ten designed primer pairs were related to the *mreB* gene of *P. puraquae*.

**FISH**

To confirm that bacteria were from inside the fungi and to identify the intracytoplasmic localization of bacterial cells, conidia and hyphae were stained and detected using FISH and confocal laser scanning microscopy. Endobacteria pre-
sented as coccoid fluorescent spots in the cytoplasm of conidia and hyphae using the EUB338 mix, probe 16S, and probe M (Fig. 3A, B, C, D, E, F, G, H, I, J, K, L, M, and N). Isolated bacteria also presented as coccoid fluorescent spots (Fig. 3O). FISH indicated that the signals stained by 6-FAM and Cy3 were endobacteria. Z-stacked images (Fig. S2, 3, 4, and 5) and time series images (Fig. S6) showed fluorescence signals in conidia and hyphae.

Ultrastructural morphology of endobacteria

To validate the molecular detection of endobacteria, TEM was used to identify the cytoplasmic locations of endobacteria within the conidia of *M. bibionidarum* CBS 648.67. Endobacteria directly embedded in the fungal cytoplasm were observed (Fig. 4) and were coccoid, similar to the endobacteria detected in *Esteya vermicola* (Wang et al., 2017). These endobacteria also had a distinct cell wall structure in the outermost layer. The endobacteria observed in TEM images were 200–500 nm in size (Fig. 4). The diameters of large endobacteria were approximately twice those of small endobacteria (Fig. 4), which indicated that large endobacteria were about to undergo cell division. This was corroborated by SEM results, as discussed below.

Fractionated endobacteria were observed by SEM. All endobacteria were spherical (Fig. 5), and their diameters in the visual field were mostly less than 500 nm. Fluorescence signals also revealed the presence of endobacteria in the isolated suspension (Fig. 3O), which indicated that the spherical structures were endobacteria. SEM images showed fiber-like structures firmly attached to the surfaces of endobacteria that were 20–40 nm in diameter (Fig. 5D and E). These fiber-like structures were suspected to be microtubules (Schwan et al., 2014; Wang et al., 2017). The central constriction (Fig. 5C, D, and E) suggested that endobacteria propagated in the cytoplasm of fungi by cell division. The diameters of the dividing endobacteria were relatively large, at approximately 500 nm.

Discussion

A combination of ultrastructural, molecular, and phylogenetic analyses demonstrated that the CBS 648.64 and CFCC 80795 strains of *Metarhizium* spp. hosted endobacteria in their conidia and hyphae. These results also showed that the presence of these bacteria was not due to exogenous bacterial contamination.

Endobacteria were abundant within fungal hyphae and conidia, as revealed by fluorescence staining using SYTO9 and FISH (Fig. 1 and 3). Their presence in both hyphae and conidia indicated that endobacteria inside CBS 648.67 were vertically transmitted from one generation to the next by sporulation (Fig. 3) and that they grew, multiplied, and migrated along with conidial germination.

The results of FISH, TEM, and SEM showed that the morphology of endobacteria within *M. bibionidarum* CBS 648.67 was coccoid. Endobacteria had cell walls with attached fibrous structural components that appeared to be microtubules rather than flagella. We speculated that similar to other fungal organelles, these endobacteria move inside the cell using microtubules (Dohner et al., 2005; Abrahams and Hensel, 2006; Wang et al., 2017). These endobacteria were smaller than the free-living close relative, *P. puraquae* (Gomila et al., 2007), which may be an adaptation to the limited living space in the fungal host.

Based on the 16S rRNA analysis, we concluded that endobacteria were closely related to *P. puraquae*, a free-living Gram-negative bacterium from the genus *Pelomonas* in the family *Comamonadaceae*. However, unlike *P. puraquae*, which is rod-shaped (Gomila et al., 2007), the endobacteria found in the present study were coccoid. The genus *Pelomonas* was previously isolated from hemo- dialysis water, industrial water, oceans, and other oligotrophic environments (Gomila et al., 2005; Gomila et al., 2006). Some strains ascribed to *P. saccharophila* act as degraders of aromatic compounds (Stringfellow and Aitken, 1995), which are contaminants of pure and ultrapure water (Kulakov et al., 2002; Gomila et al., 2005). *P. puraquae* was shown to proficiently use various carbon sources (Gomila et al., 2007). The presence of endobacteria from the genus *Pelomonas* in fungi isolated from plant seeds and leaves, as well as from grassland soils, was also confirmed in previous studies (Shaffer et al., 2016; Muller et al., 2021). Endobacteria from the genus *Pelomonas* were observed in *Pleurotus ostreatus* (Adamski and Pietr, 2019). However, the cultivability of these bacteria has not yet been demonstrated. Shaffer et al. (2018) observed the relatively powerful impact of endobacteria on shaping the effects of their fungal host Nectriaceae sp. on the viability of *Cecropia peltata* seeds. However, these effects were not solely attributed to *Pelomonas* endobacteria because of the simultaneous presence of multiple endobacteria in the fungus. Therefore, the role played by *P. puraquae* in fungi warrants further investigation.

Nitrogenase (*nifH*) and hydrogenase (*hoxG*) genes are relevant to the metabolism of the genus *Pelomonas*. The *nifH* gene is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea, and the hydrogenase-related *hoxG* gene forms a heterodimeric, membrane-bound hydrogenase with *hoxK* (Kortläke et al., 1992; Gomila et al., 2007; Gaby and Buckley, 2012). Amplification and sequencing results showed the absence of the target genes *nifH* and *hoxG* in endobacteria, which may be related to the inability of these bacteria to survive in *vitro* independently of the host fungus.

The cytoskeletal protein MreB is a bacterial ortholog of actin (Gaballah et al., 2011). It is critically involved in cell shape (Figge et al., 2004). Amplification by 11 primer pairs for the *mreB* gene and identification results showed that symbiotic bacteria lacked the *mreB* gene, which was different from free-living *Pelomonas*. The sequences amplified by the primer pair mreB-1/mreB-2 were highly similar to those of *M. brunneum* strain CP058938.1 (query cover 98%, identity 96.14%) in the NCBI database. However, FISH (Fig. 3G, H, I, J, K, L, M, and N) showed multiple fluorescence signals in single fungal cells, and the fluorescence signals of probe M were not localized to fungal nuclei, which suggested that the sequence was not present on the chromosome of the fungal host (if present, a single fluorescence signal will appear in each fungal nucleus). The fluorescence signals obtained by the hybridization of probe 16S (Fig. 3I and

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**Novel Endobacteria in *Metarhizium***
Fig. 3. Detection of endobacteria by fluorescence in situ hybridization (FISH). FISH was performed with EUB338 mix (green), probe 16S (green), probe M (red), and DAPI (blue). (A, B, C, D, E, and F) Double labeling with the universal bacterial probe EUB338 mix and DAPI. (G, H, I, J, K, L, M, and N) Triple labeling with probe 16S, probe M, and DAPI. (O) Isolated endobacteria with the probe EUB338 mix. (A, D, G, K) Superimposed images. (B, E, H, L) Blue channel. (C, F, I, M, O) Green channel. (J, N) Red channel. Hyphae (A, B, C, K, L, M, and N), isolated endobacteria (O), and conidia (D, E, G, H, I, and J). Nuclei are observed as blue fluorescence spots. Scale bars, 2 μm.
M) and probe M (Fig. 3J and N) highly overlapped, further indicating that the sequence exists in endobacteria, but not in their host. Bonfante et al. (2019) reported that endobacterial genomes contained a number of horizontally transferred genes of host fungal origin to complement the shortfall associated with gene reductions. Genome sequencing by Naito et al. (2015) provided evidence for horizontal gene transfer events, particularly fungal genes involved in post-translational modifications (Naito et al., 2015; Torres-Cortes et al., 2015). Therefore, the sequences amplified using the primers mreB-1/mreB-2 may be transcripts that endobacteria carry from the fungal host. Sequences amplified using the ten designed primer pairs were not the mreB gene of P. puraquae. Previous studies reported that marked genomic reductions and accumulated mutations during the establishment of the symbiotic relationship with the host were important factors affecting morphological and functional changes in endobacteria (McCutcheon and Moran, 2011; Bonfante and Desiro, 2017; Deveau et al., 2018; Bonfante et al., 2019). Therefore, the lack of the mreB gene and the possible horizontal transfer of genes in the endobacteria of Metarhizium strains may change the endobacterial morphology from rod-shaped to spherical, which was consistent with previous findings on symbiotic bacteria in E. vermicola (Wang et al., 2017).

Endobacteria play important roles in host morphology, host germination and growth, pathogen toxins, and nitrogen fixation, similar to mycorrhizal symbionts (Liu et al., 2019). The degradation of a host’s lipid layer, the penetration of the host cuticle, and host colonization (overcoming host defense responses) are critical invasive steps by Metarhizium spp. (Schrank and Vainstein, 2010), and are closely related to its
pathogenicity. In these steps, the production of enzymes that disrupt the integrity of the host and form toxins to overcome host defense responses result in a strong selective advantage for the pathogen. The endobacteria of *Rhizopus microsporus* have been identified as true producers of rhizoxin and ‘mycotoxin’ rhizoxin (Partida-Martinez and Hertweck, 2005, 2007). Therefore, we hypothesized that similar relationships may exist between the pathogenic ability of *Metarhizium* on insect hosts and its endobacteria. Some bacteria of the genus *Pelomonas* exhibit not only viability in oligotrophic environments, but also the utilization of multiple carbon sources (Gomila et al., 2007). Therefore, the endobacteria of *Metarhizium* may contribute to their insect host range, such as facilitating the utilization of nitrogen sources by fungi through the expansion of fungal nutrient assimilation capacities and ecological niches. These hypotheses warrant further experimental verification.

Endobacteria operate as multipliers of fungal genetic variability, providing diversity for natural selection (Bonfante et al., 2019). The host ranges and virulence levels of *Metarhizium* strains vary (Zimmermann, 2007). Research on endobacteria will provide a more detailed understanding of the phylogeny of *Metarhizium*, insights into interactions with insects and their pathogenic mechanisms, and expand the application of *Metarhizium*.

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