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Short Communication

Detection of Betaproteobacteria inside the Mycelium of the Fungus *Mortierella elongata*

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Microscopic and molecular analyses showed the presence of endobacteria inside the mycelia of four out of twelve nitrous oxide (N_2O)-producing fungal isolates identified as *Mortierella elongata*. The 16S rRNA gene was successfully amplified with DNA extracted directly from the endobacterium-containing fungal strains and all sequences were related to that of *Candidatus* Glomeribacter gigasporarum in the family *Burkholderiaceae*. Bacterial endotoxin was detected in the endobacterium-positive fungal strains but only trace levels were found in endobacterium-negative strains. No significant relationship was found between the fungal N_2O-producing activity and the presence of endobacteria.

Key words: endobacterium, N_2O production, *Mortierella elongata*, soil fungus

The intracellular distribution of bacteria, or more specifically bacterial endosymbiosis, is known for numerous eukaryotic organisms including ciliate protozoa (25), various insects (11), a frenulata (marine invertebrate) (12), and well-known legume roots. Concerning the association of bacteria with fungi, bacterial endosymbiosis has been reported in some strains of *Glomeromycota* [e.g. *Geosiphon pyriform* (22), and *Gigaspora* and *Scutellospora* species (4, 6, 7, 15)], *Ascomycota* [e.g. *Tuber borchii* (1)], *Basidiomycota* [e.g. *Laccaria bicolor*; (3)] and *Zygomycota* [e.g. *Rhizopus* species (10, 18)]. In our recent study, nitrous oxide (N_2O)-producing fungal strains were isolated from upland soils and the intramycelial localization of bacterium (endobacterium) in the fungal isolates was found (Sato, Y., *et al.* 2008. Abstracts for the 12th International Symposium on Microbial Ecology, Cairns, Australia). The purpose of this study is to examine further the presence of endobacteria using fluorescence and electron microscopy, the amount of endotoxin, the 16S rRNA gene, and the relationship between the presence of endobacteria and fungal N_2O-producing activity.

Ten fungal strains used in this study were isolated from no-tilled, low-nitrogen input cropland soils at the Field Science Center, Ibaraki University College of Agriculture, located in the Kanto plains of Japan and were identified as phylum *Zygomycota, Mortierella elongata* from their morphological characteristics and their 18S-26S/28S internal transcribed spacer sequence (ITS). All of the *M. elongata* strains were examined for activity to produce N_2O and found to have low activity levels during growth in cultures containing 10 mM nitrite but no activity in cultures with 10 mM nitrate (Y. Sato, T. Nishizawa, M. Umezu, K. Tsuruta, K. Narisawa, M. Komatsuzaki, N. Kaneko, H. Ohta, submitted for publication). Comparisons were made with herbarium materials, *Mortierella elongata* Linnemann NBRC 8570 and *M. elongata* MAFF 425591, obtained from the National Institute of Technology and Evaluation (NITE Biological Resource Center, Chiba, Japan), and National Institute of Agrobiological Sciences (NIAS Genebank, Ibaraki, Japan), respectively.

The morphology of *M. elongata* strains was observed by fluorescence microscope. Briefly, the mycelia of a culture grown for 3 days at 23°C on half-strength cornmeal-malt-yeast agar (CMMY) {grams per liter: cornmeal agar [Becton-Dickinson (BD), Franklin Lakes, NJ, USA], 8.5; malt extract (BD), 10; yeast extract (BD), 2; agar (BD), 7.5} was mounted on a polycarbonate membrane filter (black filter with 0.2 μm pore size, Advantec, Tokyo, Japan) and stained for 15 min with a LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) (8) that is specific for bacteria and can distinguish between live and dead cells. The filter was rinsed with filter-sterilized (0.2 μm pore size, Advantec) distilled water and placed on a microscope slide. Preparations were observed under blue and green light with a fluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera (DP-50, Olympus). Green-fluorescent bacteria-like organelles (BLOs) were detected inside the mycelia of strains FMR23-1 (Fig. 1A), FMR23-6, FMR23-9, and FMR13-2. Further observation showed that the BLOs existed also inside the fungal spores of all endobacterium-containing strains (data not shown). No BLOs were found in the reference strains *M. elongata* NBRC 8570 and MAFF 425591. To our knowledge, there is no report that the LIVE/DEAD *Bac*Light Bacterial Viability Kit is applicable to the mycelium of *M. elongata*. Therefore, we do not make any

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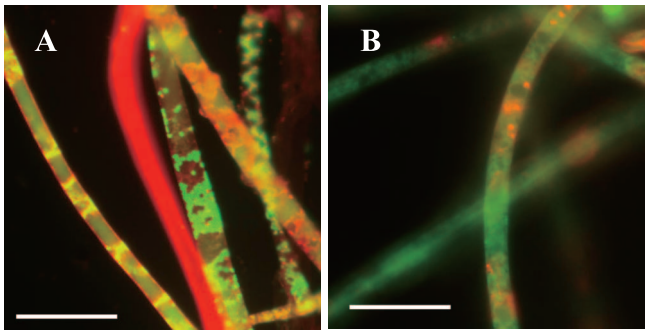

Fig. 1. Fluorescence microscopic observation of the fungal mycelia with the Live/Dead *Bac*light kit. A, green fluorescent bacterium-like organelles were found in the mycelia of *Mortierella elongata* FMR23-1; B, no bacterium-like organelle was detected in the mycelia of FMR23-3. Bars, 20 μm.

presumption as to the viability of the fungal mycelium from the image presented in Fig. 1.

To further characterize the intracellular distribution of the bacteria, ultrathin sections of the mycelia were prepared and examined with transmission electron microscopy. The growing mycelium of *M. elongata* FMR23-6 grown for 3 days at 23°C on CMMY was fixed with 3.5% glutaraldehyde in 1/15 M potassium phosphate buffer (pH 7.0) for 1 h at room temperature. After further fixing with a 2% (w/v) osmium tetraoxide (OsO_4) solution for 1 h at room temperature, the sample was embedded in Spurr's resin (24). Ultrathin sections were obtained with a glass knife on an ultramicrotome Ultracut UCT (Leica Microsystems, Wetzlar, Germany), stained with 0.5% (w/v) potassium permanganate ($KMnO_4$) in a 1/15 M potassium phosphate buffer (pH 7.0) for 1 min, and rinsed with a 0.05% (w/v) citric acid solution for 10 s, then post-stained with lead citrate (20) for 5 min and observed under a Hitachi H-7600 transmission electron microscope (Hitachi, Tokyo, Japan) at 100 kV.

Transmission electron microscopy revealed that *M. elongata* strain FMR23-6 formed intrahyphal hyphae inside the cytoplasm in the primary mycelium (Fig. 2A). This unique structure was already reported for *Mortierella multidivaricata*, in a subgenus, *Gamsiella*, of *Mortierella* (2). According to the description, the intrahyphal hyphae arise not infrequently from the end of the living cell segment of the ageing aerial mycelium of *M. multidivaricata* (2). The BLOs were observed in the cytoplasm of primary hyphae and some BLOs were attached to secondary hyphae of *M. elongata* FMR23-6 (Fig. 2A). A magnified image of endobacteria in the fungal cytoplasm showed that they possessed a double-layered cell envelope and cytoplasm rich in ribosomes (Fig. 2B). These microscopic results suggest the presence of endobacteria, probably double-layered Gram-negative bacteria, inside the mycelia of the *M. elongata* strains.

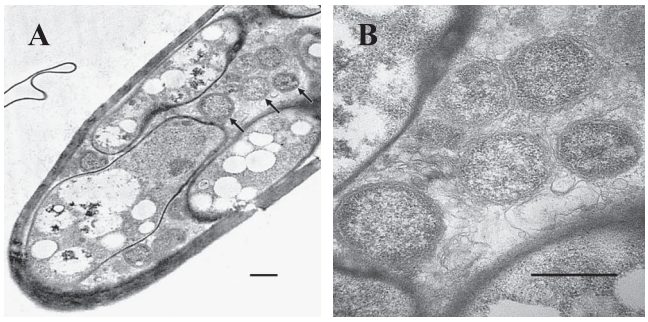

Fig. 2. Transmission electron micrographs of endobacteria inside the fungal mycelia of *Mortierella elongata* FMR23-6. (A) Endobacteria (arrows) present in the fungal cytoplasm. Bar, 0.5 μm. (B) Magnification of endobacteria with a layered cell envelope. Bar, 0.5 μm.

To characterize the endobacteria, PCR amplification was carried out in a reaction mixture containing 100 ng of template DNA extracted from fungal mycelia using the ISOPLANT DNA extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The primers for the bacterial 16S rRNA gene were 10F primer (5'-AGTTTGATATCCTGGCTCAG-3', corresponding to positions 10–27 of the *E. coli* 16S rRNA gene) and 1541R primer (5'-AAGGAGGTGATCCAGCCG-3', positions 1524–1541). The reaction profile was as follows: initial denaturation at 95°C for 5 min followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 1 min. Amplified fragments (approximately 1.5 kb) were obtained from FMR23-1, FMR23-6, FMR23-9 and FMR13-2, while no 16S rRNA gene fragment was amplified from the other 10 strains using universal bacterial primers (Table 1).

To check the endobacterial diversity associated with the single fungal strain, terminal restriction fragment length polymorphism (T-RFLP) fingerprinting was conducted as described previously (21). Briefly, the PCR for T-RFLP fingerprinting (14) was performed using a 5'-end fluorescence (BODIPY FL)-labeled primer (Q-10F; *E. coli* positions 10–27) purchased from J–Bio21 (Tsukuba, Japan) and 926r (*E. coli* positions 907–926) under conditions of 2 min at 95°C, then 15 cycles of 95°C (30 s), 54°C (45 s), and 72°C (1.5 min). The fluorescently labeled-PCR products were purified using a Qiagen DNA purification Kit (Qiagen, Hilden, Germany) and eluted in a final volume of 30 μL. Aliquots (5 μL) of the amplified product were separately digested with *Hae*III, *Msp*I, and *Rsa*I (Takara Bio, Otsu, Japan) for 2 h according to the manufacturer's instructions. The labeled fragments were purified with the Qiagen DNA purification kit and separated on the Applied Biosystems 3130*xl* DNA Sequencer

Table 1. The PCR amplification and *Limulus* amebocyte lysate test for detecting endobacteria in the mycelia of *Mortierella elongata* strains.

Strain (accession number)	PCR amplification of 16S rRNA gene*	Et concentration (μg Et g wet mycelia^{-1})
FMR23-1 (AB542099)	+	70
FMR23-2 (AB542100)	–	<0.1
FMR23-3 (AB542101)	–	0.4
FMR23-6 (AB542104)	+	240
FMR23-9 (AB542107)	+	420
FMR23-10 (AB542108)	–	<0.1
FMR13-1 (AB542092)	–	<0.1
FMR13-2 (AB542093)	+	250
FMR13-3 (AB542094)	–	0.3
FMR13-5 (AB542096)	–	<0.1
MAFF 425591 (AB542112)	–	<0.1
NBRC 8570 (AB542111)	–	<0.1

*+, positive reaction; –, negative reaction

in the GeneScan mode (Applied Biosystems, Foster, CA, USA). The lengths of fluorescently labeled T-RFs were determined by GeneMapper software (ver. 3.7). Based on the determined endobacterial 16S rRNA gene sequences from strains FMR23-1, FMR23-6, FMR23-9 and FMR13-2, the sizes of the deduced T-RFs were respectively, 75, 75, 202 and 75 for the *Hae*III-digests, 65, 65, 209, and 205 for the *Hha*I-digests, 139, 139, 143 and 139 for the *Msp*I-digests, and 471, 471, 475 and 471 for the *Rsa*I-digests. Single T-RF with the above deduced size was detected in each digest of the PCR products, suggesting that each of the endobacterium-positive *M. elongata* strains harbored an identical bacterium with respect to the sequence of the 16S rRNA gene.

The phylogenetic positions of endobacterial 16S rRNA genes from strains FMR23-1, FMR23-6, FMR23-9 and FMR13-2 were determined as described previously (accession number, AB558491 to AB558494) (21). As shown in Fig. 3, a phylogenetic tree based on 16S rRNA gene sequences showed that all endobacteria from different strains of *M. elongata* belonged to the family *Burkholderiaceae* in the *Betaproteobacteria* with the closest neighbors of genera *Candidatus* Glomeribacter, *Burkholderia*, and *Pandoraea*. The 16S rRNA gene sequences (1,422–1,440 bp) of the two endobacteria from fungal strains FMR23-1 and FMR23-6 were clustered together with high similarity (99.8%). On the other hand, the other two endobacteria from strains FMR13-2 and FMR23-9 were clustered separately from those from strains FMR23-1 and FMR23-6: 91.4% similarity for the pair of endobacteria from FMR23-1/FMR23-9 and 94.3% for the pair from FMR23-1/FMR13-2. Our endobacteria were distantly placed from the known genera, *Candidatus* Glomeribacter, *Burkholderia*, and *Pandoraea*. According to studies on uncultured bacteria (16, 17), further morphological and genomic characterization is required to assign our endobacteria to new *Candidatus* positions.

Interactions between *Burkholderia* strains and fungal strains have been reported in several studies. For example,

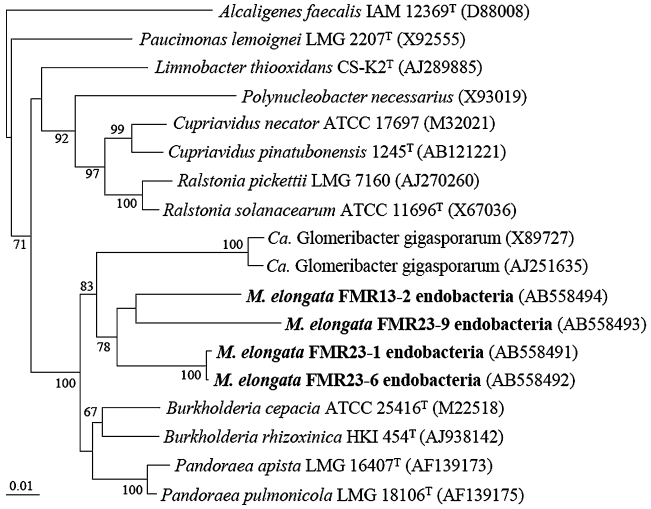

Fig. 3. Neighbor-joining unrooted tree based on 16S rRNA gene sequences (1,422–1,440 bp), showing the phylogenetic relationship between the endobacteria from the *M. elongata* strains (AB558491–AB558494), other members of the family *Burkholderiaceae*, and *Alcaligenes faecalis* IAM 12369^T in the family *Alcaligenaceae*. Values along branches indicate bootstrap percentages of >60%, based on 1,000 resamplings. Bar, 0.01 substitutions per nucleotide position.

Burkholderia fungorum, *Burkholderia sordidicola*, and *Burkholderia cepacia* complex have been found in association with white-rot fungi, *Phanerochaete chrysosporium*, *Phanerochaete sordida*, and *Pleurotus ostreatus*, respectively (9, 13, 23, 26). *Burkholderia rhizoxinica* and *Burkholderia endofungorum* were detected inside the mycelia of *Rhizopus microspores* (19). Uncultured endobacteria of *Candidatus* Glomeribacter gigasporarum, formerly classified as *Burkholderia*, were detected in an arbuscular mycorryhizal fungus, *Gigaspora margarita* (5). In relation to fungal growth and activity, the biological and metabolic functions of these endobacteria are not yet known.

The *Limulus* amebocyte lysate (LAL) test was performed using the fungal mycelia to detect bacterial endotoxin (Et). The growing mycelia (5.0 mg) of *M. elongata* strains grown for 3 days at 23°C on CMMY were collected with needles and placed into sterilized eppendolf tubes containing 500 µL of sterilized MilliQ water and approximately 0.1 g each of glass beads (1.0, 0.5, and 0.1 mm in diameter). The mycelia were broken down by vortex mixer for 30 min and 10 µL of supernatant was transferred to a new tube. Then the supernatant was diluted with 990 µL of sterilized MilliQ water. Et was determined using LAL test reagents (Endospecy ES-50M, Seikagaku, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 50 µL each of the samples and serial dilutions of standard Et solution from *E. coli* O113:H10 (Seikagaku) were put into each well of a 96-well plate, and mixed with an equal volume of the LAL solution. After mixing for 30 s on a plate mixer, absorbance was measured at 405 nm (reading wavelength) and 490 nm (reference wavelength) from 0 to 30 min at 37°C on a 1420 ARVOsx multilabel counter (Wallac, Turku, Finland). The Et concentrations were calculated based on the standard Et solution. All equipment used in this procedure was sterilized at 250°C for 30 min by dry heat sterilizers. As presented in Table 1, significantly higher values were obtained in the endobacterium-positive strains FMR23-1 (70 µg Et g wet mycelia^{-1}), FMR23-6 (240 µg Et g wet mycelia^{-1}), and FMR23-9 (420 µg Et g wet mycelia^{-1}) and FMR13-2 (250 µg Et g wet mycelia^{-1}) than the 10 endobacterium-negative strains (ranging from <0.1 to 0.5 µg Et g wet mycelia^{-1}).

Finally, strain FMR23-6 was selected for examining the relationship between the presence of endobacteria and the fungal activity for N_2O production. Prior to this examination, to generate the endobacterium-free strain, fifty single spores were isolated from FMR23-6. The PCR amplification and the LAL test described above revealed that two of the fifty offspring (FMR23-6-II-A1 and FMR23-6-II-A4) did not contain detectable levels of endobacteria. These endobacterium-free variants and the parent strain were examined for N_2O-producing activity by growing them at 30°C in butyl rubber-stoppered test tubes (50 mL volume) containing 13.5 mL of glycerol medium. The medium contained (per liter): glycerol, 30 mL; peptone (BD), 2 g; KH_2PO_4, 0.68 g; K_2HPO_4, 0.87 g; $FeCl_3$, 3.6 mg; $FeSO_4 \cdot 7H_2O$, 0.06 mg; $CuSO_4 \cdot 5H_2O$, 1.6 mg; $CaCl_2 \cdot 2H_2O$, 1.6 mg; $CoCl_2 \cdot 2H_2O$, 0.16 mg; $NaMoO_4$, 1.6 mg. The medium was supplemented with 10 mM $NaNO_2$. Triplicate cultures were made for each strain and incubated for 10 days. Fungus-free control cultures were incubated in the same manner to measure the background level of N_2O. The nitrous oxide in the headspace

of culture tubes was determined by a Shimadzu GC-8AIE gas chromatograph equipped with a ^{63}Ni electron capture detector (Shimadzu, Kyoto, Japan) and a 2-m stainless steel column (3.0-mm diameter) with Porapak Q (80–100 mesh). The amount of N_2O produced was expressed by subtracting the background level from the measured value for each culture. The activities of strain FMR23-6 and the two endobacterium-free variants, FMR23-6-II-A1 and FMR23-6-II-A4, were 52, 67 and 96 ng N_2O-N formed tube^{-1} day^{-1} (median, 19 ng N_2O-N formed tube^{-1} day^{-1}), respectively. In our previous study, activity for N_2O production from nitrite was found in several endobacterium-negative strains of *M. elongata* (Y. Sato, T. Nishizawa, M. Umezu, K. Tsuruta, K. Narisawa, M. Komatsuzaki, N. Kaneko, H. Ohta, submitted for publication). Therefore, it can be concluded that the N_2O production by the *M. elongata* isolates originated from fungal metabolism.

In our experiments, the fungal strains were grown for 3 days and the viability of the fungal cells seemed to be very high. Yet, it may be assumed that endobacteria occurred in a few dead mycelia of the total population as a result of bacterial attack and invasion. To examine this further, an accurate determination of fungal cell viability will be needed. In conclusion, this study has shown that several strains of a N_2O-producing fungus *Mortierella elongata* harbored endobacteria belonging to the family *Burkholderiaceae* in the *Betaproteobacteria*. The ecological and metabolic significance of the association of *Mortierella elongata* strains with the *Burkholderia*-related endobacteria remains to be studied.

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References

1. Barbieri, E., L. Potenza, I. Rossi, D. Sisti, G. Giomaro, S. Rossetti, C. Beimfohr, and V. Stocchi. 2000. Phylogenetic characterization and in situ detection of a Cytophaga-Flexibacter-Bacteroides phylogroup bacterium in *Tuber borchii* vittad. ectomycorrhizal mycelium. Appl. Environ. Microbiol. 66:5035–5042.
2. Benjamin, R.K. 1978. *Gamsiella*, a new subgenus of *Mortierella* (Mucorales: Mortierellaceae). Aliso 9:157–170.
3. Bertaux, J., M. Schmid, N.C. Prevost-Boure, J.L. Churin, A. Hartmann, J. Garbaye, and P. Frey-Klett. 2003. In situ identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. Appl. Environ. Microbiol. 69:4243–4248.
4. Bianciotto, V., C. Bandi, D. Minerdi, M. Sironi, H.V. Tichy, and P. Bonfante. 1996. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl. Environ. Microbiol. 62:3005–3010.
5. Bianciotto, V., E. Lumini, L. Lanfranco, D. Minerdi, P. Bonfante, and S. Perotto. 2000. Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family Gigasporaceae. Appl. Environ. Microbiol. 66:4503–4509.
6. Bianciotto, V., E. Lumini, P. Bonfante, and P. Vandamme. 2003. '*Candidatus* glomeribacter gigasporarum' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi. Int. J. Syst. Evol. Microbiol. 53:121–124.
7. Bonfante, P., R. Balestrini, and K. Mendgen. 1994. Storage and secretion processes in the spore of *Gigaspora margarita* Becker & Hall as revealed by high-pressure freezing and freeze substitution. New Phytol. 128:93–101.
8. Boulos, L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins. 1999. LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods 37:77–86.
9. Coenye, T., S. Laevens, A. Willems, M. Ohlen, W. Hannant, J.R. Govan, M. Gillis, E. Falsen, and P. Vandamme. 2001. *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. Int. J. Syst. Evol. Microbiol. 51:1099–1107.
10. Ibrahim, A.S., T. Gebremariam, M. Liu, *et al.* 2008. Bacterial endosymbiosis is widely present among zygomycetes but does not contribute to the pathogenesis of mucormycosis. J. Infect. Dis. 198:1083–1090.
11. Kikuchi, Y. 2009. Endosymbiotic bacteria in insects: their diversity and culturability. Microbes Environ. 24:195–204.
12. Kubota, N., M. Kanemori, Y. Sasayama, M. Aida, and Y. Fukumori. 2007. Identification of endosymbionts in *Oligobrachia mashikoi* (Siboglinidae, Annelida). Microbes Environ. 22:136–144.
13. Lim, Y.W., K.S. Baik, S.K. Han, S.B. Kim, and K.S. Bae. 2003. *Burkholderia sordidicola* sp. nov., isolated from the white-rot fungus *Phanerochaete sordida*. Int. J. Syst. Evol. Microbiol. 53:1631–1636.
14. Liu, W.T., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516–4522.
15. MacDonald, R.M., M.R. Chandler, and B. Mosse. 1982. The occurrence of bacterium-like organelles in vesicular-arbuscular mycorrizal fungi. New Phytol. 90:659–663.
16. Murray, R.G., and K.H. Schleifer. 1994. Taxonomic notes: a proposal for recording the properties of putative taxa of procaryotes. Int. J. Syst. Bacteriol. 44:174–176.
17. Murray, R.G., and E. Stackebrandt. 1995. Taxonomic note: implementation of the provisional status Candidatus for incompletely described procaryotes. Int. J. Syst. Bacteriol. 45:186–187.
18. Partida-Martinez, L.P., and C. Hertweck. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437:884–888.
19. Partida-Martinez, L.P., I. Groth, I. Schmitt, W. Richter, M. Roth, and C. Hertweck. 2007. *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. Int. J. Syst. Evol. Microbiol. 57:2583–2590.
20. Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208–212.
21. Sato, Y., K. Hosokawa, R. Fujimura, T. Nishizawa, T. Kamijo, and H. Ohta. 2009. Nitrogenase activity (acetylene reduction) of an iron-oxidizing *Leptospirillum* strain cultured as a pioneer microbe from a recent volcanic deposit on Miyake-jima, Japan. Microbes Environ. 24:291–296.
22. Schussler, A., D. Mollenhauer, E. Schnepf, and M. Kluge. 1994. *Geosiphon pyriforme*, an endosymbiotic association of fungus and cyanobacteria: the spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot. Acta 107:36–45.
23. Seigle-Murandi, F., P. Guiraud, J. Croize, E. Falsen, and K.L. Eriksson. 1996. Bacteria are omnipresent on *Phanerochaete chrysosporium* Burdsall. Appl. Environ. Microbiol. 62:2477–2481.
24. Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31–43.
25. Stevenson, I. 1972. Bacterial endosymbiosis in *Paramecium aurelia*: bacteriophage-like inclusions in a kappa symbiont. J. Gen. Microbiol. 71:69–76.
26. Yara, R., W. Maccheroni, Jr., J. Horii, and J.L. Azevedo. 2006. A bacterium belonging to the *Burkholderia* cepacia complex associated with *Pleurotus ostreatus*. J. Microbiol. 44:263–268.