Mycoavidus cysteinexigens gen. nov., sp. nov., an endohyphal bacterium isolated from a soil isolate of the fungus *Mortierella elongata*

Shoko Ohshima,[1] Yoshinori Sato,[2] Reiko Fujimura,[3] Yusuke Takashima,[1,4] Moriyuki Hamada,[5] Tomoyasu Nishizawa,[1] Kazuhiko Narisawa[1,4] and Hiroyuki Ohta[1,4]

Correspondence
Hiroyuki Ohta
hiroyuki.ohta.1494@vc.ibaraki.ac.jp

[1]Ibaraki University College of Agriculture, 3-21-1 Chuo, Ami-machi, Ibaraki 300-0393, Japan

[2]National Research Institute for Cultural Properties, Tokyo, 13-43 Ueno-park, Taito-ku, Tokyo 110-8713, Japan

[3]Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5, Kashiwanoha, Kashiwa-shi, Chiba 277-8564, Japan

[4]United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan

[5]Biological Resource Center, National Institute of Technology and Evaluation (NBRC), 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818, Japan

An endohyphal bacterium (strain B1-EB^T) living in association with the fungus *Mortierella elongata* FMR23-6 I-B1 was isolated from a fungal cell homogenate and studied for its taxonomic allocation. Cells were Gram-stain-negative, rod-shaped, non-spore-forming, non-motile, and negative for oxidase and catalase. Strain B1-EB^T required cysteine for growth and grew at temperatures between 4 and 35 °C. A comparative analysis of 16S rRNA gene sequences revealed that strain B1-EB^T forms a distinct clade in the family *Burkholderiaceae*, encompassing a group of endosymbionts associated with several soil isolates of *M. elongata*. The most closely related genus is '*Candidatus* Glomeribacter gigasporarum', an endosymbiont of the arbuscular mycorrhizal fungus *Gigaspora margarita*. The major cellular fatty acids of strain B1-EB^T were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and $C_{16:1}\omega6c$) and summed feature 8 ($C_{18:1}\omega7c$ or $C_{18:1}\omega6c$). Ubiquinone Q-8 was the only quinone detected. The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, an unknown aminophospholipid and two unknown aminolipids. The DNA G+C content was 49.8 mol%. On the basis of phenotypic, chemotaxonomic, and phylogenetic characteristics, strain B1-EB^T represents a novel genus and novel species in the family *Burkholderiaceae*, for which the name *Mycoavidus cysteinexigens* gen. nov., sp. nov. is proposed. The type strain is B1-EB^T (=JCM 30646^T=LMG 28693^T=NBRC 110909^T).

Bacterial endosymbionts (endobacteria) present in fungi were first noted in an arbuscular mycorrhizal (AM) fungus in 1970 (Mosse, 1970). The range of fungi known to have endobacteria has expanded to include not only various AM fungi in the Glomeromycota (Desirò *et al.*, 2014; Naumann *et al.*, 2010) but also the Ascomycota (Barbieri *et al.*, 2000; Hoffman & Arnold, 2010), Basidiomycota (Sharma *et al.*, 2008) and Mucoromycotina (Ibrahim *et al.*, 2008; Kai *et al.*, 2012; Lackner *et al.*, 2009; Sato *et al.*, 2010). Although many of these endobacteria have not yet been cultured, an endobacterium found in an AM fungus, *Gigaspora margarita*, was identified as a novel betaproteobacterium, '*Candidatus* Glomeribacter gigasporarum' (Bianciotto *et al.*, 2003). Since then, two endobacteria have been successfully isolated from the phytopathogenic fungus *Rhizopus microsporus* and described as representatives of novel taxa, *Burkholderia rhizoxinica* and *Burkholderia endofungorum*, in the class *Betaproteobacteria* (Partida-Martinez *et al.*, 2007).

In our previous study, bacteria living in association with the soil fungus *Mortierella elongata* were characterized

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B1-EB^T is LC005489.

Three supplementary figures are available with the online Supplementary Material.

morphologically by fluorescence and electron microscopy. They were further identified as a novel group belonging to the family *Burkholderiaceae* by PCR-based 16S rRNA gene analysis of the fungal homogenates (Sato *et al.*, 2010). Terminal RFLP (T-RFLP) fingerprinting revealed that each of the *M. elongata* isolates possessed an identical bacterium with respect to the sequence of the 16S rRNA gene (Sato *et al.*, 2010). Our previous attempts to isolate and culture the fungus-associated bacteria on conventional nutrient media were unsuccessful. Therefore, to identify their metabolic requirements, the bacterial fraction was prepared from the fungal homogenate of *M. elongata* FMR23-6 and subjected to whole-genome analysis. The results revealed that the fungus-associated bacterium lacked several key genes responsible for cysteine biosynthesis as well as the glycolytic pathway (Fujimura *et al.*, 2014). Based on this finding, we decided to try to use the cysteine-containing buffered charcoal-yeast extract agar (Feeley *et al.*, 1979) supplemented with 0.1 % (w/v) 2-oxoglutalate (B-CYEα) to isolate the fungus-associated bacterium. This attempt resulted in a successful isolation and the isolate was named B1-EB^T. Here we describe the phenotypical, chemotaxonomic and phylogenetic characteristics of *Mycoavidus cysteinexigens* gen. nov., sp. nov. with B1-EB^T as the type strain.

The presence of endohyphal bacteria in the fungus *M. elongata* FMR23-6 I-B1, derived from the original *M. elongata* FMR23-6, was confirmed by fluorescence *in situ* hybridization (FISH) microscopy as described by Kai *et al.* (2012), with a minor modification, using a 1.5 ml tube for hybridization. The endohyphal bacteria were detected not only by the universal bacterial probe EUB338 (Amann *et al.*, 1990) but also the probe CaGgADf1, designed to detect '*Ca.* Glomeribacter gigasporarum' (Desirò *et al.*, 2014) (Fig. S1, available in the online Supplementary Material). Prior to the FISH experiments, the sequence specificity of the probe CaGgADf1 to the endohyphal bacterium of *M. elongata* FMR23-6 I-B1 was confirmed by the Silva RNA database using TestProbe 3.0 (*http://www.arb-silva.de/search/testprobe/*) with Silva SSU r123 databases (Quast *et al.*, 2013).

To isolate the endohyphal bacteria, the fungus *M. elongata* strain FMR23-6 I-B1 was cultivated for 7 days at 23 °C on half-strength cornmeal-malt-yeast (CMMY) agar (per litre distilled water): 8.5 g cornmeal agar; 10 g malt extract; 2 g yeast extract; and 7.5 g agar (sourced from Becton Dickinson). Cultivated fungal hyphae were homogenized by sterilized glass beads on a vortex mixer for 5 min and centrifuged at $1800 \times \boldsymbol{g}$ for 10 min. The supernatant was filtered through 8-µm- and then 3-µm-pore membrane filters to remove fragmented hyphae and sporangiospores. Aliquots of the filtered suspension were spread on B-CYEα agar [per litre distilled water: 10 g yeast extract; 2 g charcoal powder; 0.4 g L-cysteine hydrochloride; 0.25 g ferric pyrophosphate (soluble); 10 g ACES; 1 g potassium 2-oxoglutarate; and 15 g agar at pH 6.9, sourced from Eiken Chemical] and incubated for 7 days at 30 °C. After a purification step, where a single colony was transferred onto a fresh B-CYEα agar plate, strain B1-EB^T was obtained.

Phenotypic tests, including Gram reaction, motility, oxidase and catalase tests, were performed as previously described (Ohta & Hattori, 1983). Morphological characteristics were observed by transmission electron microscopy [JEM-1200EX (JEOL) at the Hanaichi Ultra-Structure Research Institute]. Biochemical analysis was conducted using the API 20 NE and API ZYM kits (bioMérieux) according to the manufacturer's instructions. Growth at different temperatures (4, 10, 23, 30, 32, 34, 35, 37 and 42 °C) was tested on B-CYEα agar plate medium at pH 6.9. To examine the strain's cysteine requirement, B-CYEα agar medium (CM655 and SR110; Kanto Chemical) and B-CYEα agar medium without L-cysteine (CM655 and SR175; Kanto Chemical) were used. Growth was monitored for the 7 days of incubation and the cysteine requirement was assessed by comparing growth in the presence and absence of cysteine. Anaerobic and microaerobic [6–12 % (v/v) oxygen] culturing were performed using O_2-absorbing and CO_2-generating agents (AnaeroPack-Anaero and AnaeroPack-MicroAero; Mitsubishi Gas Chemical).

For the analysis of cellular fatty acids, strain B1-EB^T was cultivated on B-CYEα agar plate medium (Eiken Chemical) for 3 days at 30 °C. Cellular fatty acid methyl esters were prepared by heating dried cells in anhydrous methanolic HCl at 100 °C for 3 h and were then analysed by GC [7890A GC system (Agilent) at TechnoSuruga Laboratory] according to the instructions of the Sherlock Microbial Identification System version 6.0 (MIDI). Fatty acid methyl ester peaks were identified, based on the TSBA6 database. Polar lipids were extracted from 100 mg of

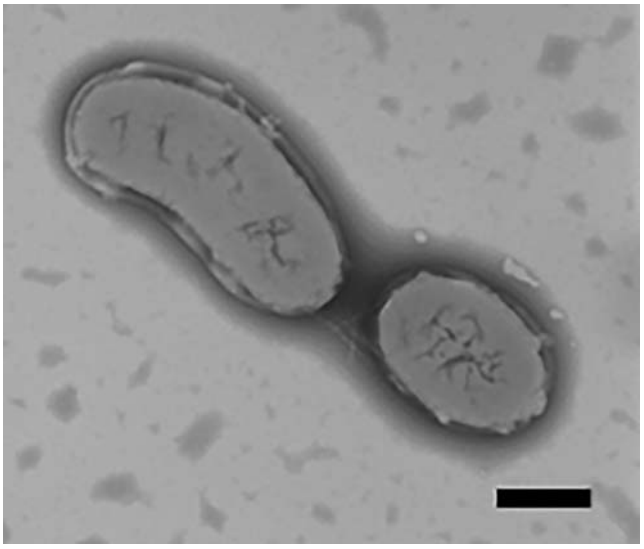

Fig. 1. Transmission electron micrograph of negative-staining cells of strain B1-EB^T. Bar, 400 nm.

freeze-dried cells using the method described by Minnikin *et al.* (1979) and analysed by TLC using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first direction and chloroform/acetic acid/methanol/water (80 : 18 : 12 : 5, by vol.) in the second. Polar lipids were visualized by spraying the TLC plate with 5 % molybdophosphoric acid. Dittmer and Lester reagent (phosphorus), ninhydrin (amino group), Schiff's reagent (glycol) and anisaldehyde (sugar) were also used as specific spray reagents for polar lipids. Isoprenoid quinones were extracted and analysed by reverse-phase TLC. The DNA G+C content was analysed by the HPLC method (Tamaoka & Komagata, 1984) at TechnoSuruga Laboratory.

To analyse the taxonomic position of strain B1-EB^T, an almost-complete 16S rRNA gene sequence was determined by the conventional protocol (Sato *et al.*, 2010). The ~1500 bases of the 16S rRNA gene nucleotide sequence were aligned by MUSCLE v3.8.31 (Edgar, 2004). Bayesian phylogenetic inference was conducted using MrBayes v.3.2.5 (Huelsenbeck & Ronquist, 2001) using the Markov Chain Monte Carlo (MCMC) approach. One tree was sampled per 1000 trees generated, and consensus topology and the best posterior probability was obtained after 76 000 trees were generated. The Average Standard Deviation of Split Frequencies (ASDSF) value was <0.01 and the Average Potential Scale Reduction Factor (APSRF) value was

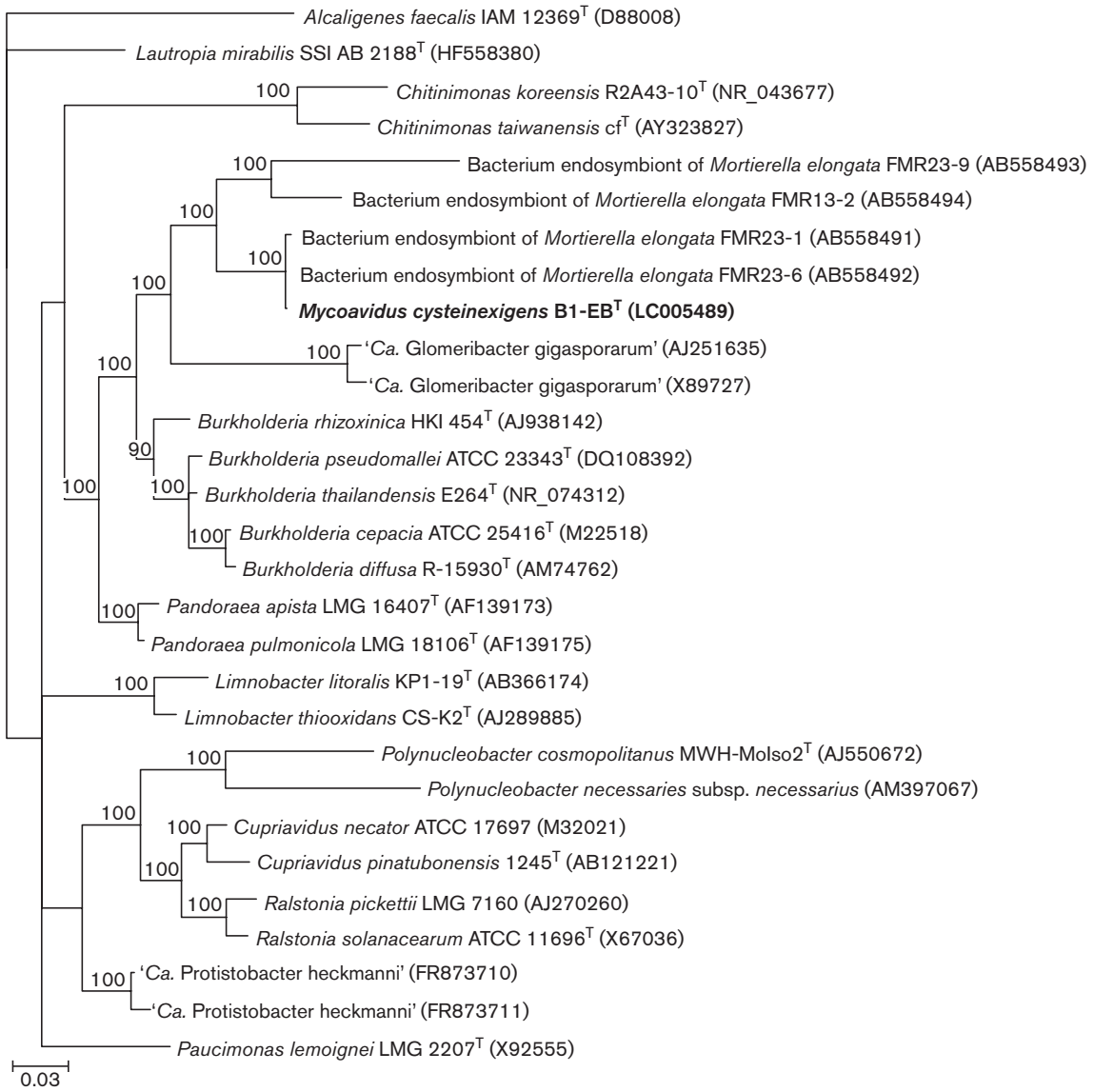

Fig. 2. Bayesian phylogenetic tree based on a 1485-position 16S rRNA gene sequence alignment, showing relationships between strain B1-EB^T and related taxa within the family *Burkholderiaceae*. Numbers at nodes are posterior probability values (%); values lower than 90 % are not shown. Bar, 0.03 nucleotide substitutions per position. The sequence of *Alcaligenes faecalis* IAM 12369^T was used as an outgroup.

1.004. The phylogram was visualized using FigTree v. 1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree/).

The colonies of strain B1-EB^T were flat, irregular, viscous and white to cream colour on B-CYEα agar plates. Cells of strain B1-EB^T were non-motile, Gram-stain-negative, short rods (1.2–1.8 μm long and 0.5–0.7 μm wide; Figs 1 and S2) that were negative for oxidase and catalase. Subcultures of strain B1-EB^T on B-CYEα medium were successful and visible growth was observed at 3 days of incubation only when a large inoculum was used. Strain B1-EB^T grew under aerobic and microaerobic conditions, but not in anaerobic conditions. Growth was found at temperatures between 4 and 35 °C (temperatures below 4 °C were not tested) on B-CYEα agar plates. Growth of strain B1-EB^T was not observed on the B-CYEα agar medium without L-cysteine after 7 days of incubation at 30 °C. As far as we tested, the strain could only be cultured on a B-CYEα agar plate at pH 6.9, and hence the levels of growth at different pH were not tested. Other results from the phenotypic tests for strain B1-EB^T are provided in the species description.

The major cellular fatty acids (>5.0 %) of three-day-cultured cells were $C_{16:0}$ (23.2 %), summed feature 8 ($C_{18:1}\omega7c$ or $C_{18:1}\omega6c$; 26.6 %), summed feature 3 ($C_{16:1}\omega7c$ and $C_{16:1}\omega6c$; 23.7 %), $C_{16:1}$ 2-OH (8.5 %) and summed feature 2 ($C_{14:0}$ 3-OH and $C_{16:1}$ iso I; 5.1 %), and other minor fatty acids (<5.0 %) were $C_{16:0}$ 3-OH (2.4 %), $C_{14:0}$ (2.3 %), $C_{18:1}$ 2-OH (2.2 %), $C_{16:0}$ 2-OH (2.0 %) and $C_{12:0}$ (1.3 %). The principal polar lipids of strain B1-EB^T were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, an unknown aminophospholipid and two unknown aminolipids. Minor or trace amounts of another aminolipid and three unidentified lipids were also detected (Fig. S3). The only quinone component detected was ubiquinone-8 (Q-8) and the DNA G+C content was 49.8 mol%.

Comparative 16S rRNA gene sequence analysis confirmed that strain B1-EB^T showed the highest 16S rRNA gene sequence similarity with 'Bacterium endosymbiont of *M. elongata* FMR23-6' (sequence similarity, 100 %; accession number, AB558492) and 'Bacterium endosymbiont of *M. elongata* FMR23-1' (99.8 %; AB558491) (Sato *et al.*, 2010). The Bayesian phylogenetic tree from a 1485-position 16S rRNA gene sequence alignment revealed that strain B1-EB^T formed a distinct clade with other yet-uncultured endosymbionts of *M. elongata* FMR13-2 and FMR23-9 (Fig. 2). The most closely related genera were '*Candidatus* Glomeribacter' and *Burkholderia* in the family *Burkholderiaceae* with sequence similarity values of <94.3 % (Fig. 2). In the phylogenetic tree, the genus *Thermothrix* described in the family *Burkholderiaceae* was excluded because the 16S rRNA gene sequence of the type strain of the type species, *Thermothrix thiopara*, is known to show an unexpected affiliation with the family *Aquificaceae* (from: *http://www.bacterio.net/thermothrix.html*, 'List of Prokaryotic Names with Standing in Nomenclature'). The major characteristics that differentiate strain B1-EB^T from the type strains of the genera in the family *Burkholderiaceae* are summarized in Table 1.

On the basis of the data presented in this study, strain B1-EB^T represents a novel species in a new genus in the family *Burkholderiaceae*, for which the name *Mycoavidus cysteinexigens* gen. nov., sp. nov. is proposed.

Description of *Mycoavidus* gen. nov.

Mycoavidus (My.co.a.vi′dus. Gr. n. *mukês -etis* a mushroom, fungus; L. adj. *avidus* eager for, loving; N.L. masc. n. *Mycoavidus* fungus-lover).

Cells are Gram-stain-negative, non-motile short rods. Negative for catalase and oxidase. The major cellular fatty acids are $C_{16:0}$, summed feature 8 ($C_{18:1}\omega7c$ or $C_{18:1}\omega6c$) and summed feature 3 ($C_{16:1}\omega7c$ and $C_{16:1}\omega6c$). The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, as well as unknown aminolipids and aminophospholipids. The predominant

Table 1. Differential characteristics of strain B1-EB^T and genera in the family *Burkholderiaceae*

Taxa: 1, B1-EB^T; 2, *Burkholderia* (Gillis *et al.*, 1995; Yabuuchi *et al.*, 1992); 3, *Pandoraea* (Anandham *et al.*, 2010; Coenye *et al.*, 2000; Sahin *et al.*, 2011); 4, *Lautropia* (Daneshvar *et al.*, 2001; Gerner-Smidt *et al.*, 1994); 5, *Limnobacter* (Lu *et al.*, 2011; Spring *et al.*, 2001); 6, *Cupriavidus* (Vandamme & Coenye, 2004; Vaneechoutte *et al.*, 2004); 7, *Ralstonia* (Coenye *et al.*, 2003; Yabuuchi *et al.*, 1992); 8, *Paucimonas* (Jendrossek, 2001); 9, *Chitinimonas* (Chang *et al.*, 2004; Joung *et al.*, 2014; Kim *et al.*, 2006; Li *et al.*, 2014); 10, *Polynucleobacter* (Hahn *et al.*, 2009; Hahn *et al.*, 2012). Because of the unexpected affiliation with the family *Aquificaceae* (from: *http://www.bacterio.net/thermothrix.html*, 'List of Prokaryotic names with Standing in Nomenclature'), the genus *Thermothrix* described in the family *Burkholderiaceae* is excluded from the table.

Characteristic	1	2	3	4	5	6	7	8	9	10
Motility	−	+/−	+	+	+	+/−	+/−	+	+	−
Oxidase	−	+/−	+/−	+	+	+	+	+	+/−	+
Catalase	−	+	+/−	+	+	+	+	+	+/−	+
Growth at 41 °C	−	+/−*	+/−*	+	+/−	+/−	+/−	−	+/−	−
DNA G+C content (mol%)	49.8	59.0–69.5	61.9–65.8	64.6–65.4	55–59	63–69	63.9–66.6	59±2	59.8–65.0	40.3–49.4

*Growth at 42 °C.

quinone is Q-8. The DNA G+C content of the type strain is 49.8 mol%. Phylogenetically, the genus *Mycoavidus* is a member of the family *Burkholderiaceae* in the class *Betaproteobacteria*. The type species is *Mycoavidus cysteinexigens*. Known habitat is associated with fungi.

Description of *Mycoavidus cysteinexigens* sp. nov.

Mycoavidus cysteinexigens (cys.te.in.ex′i.gens. N.L. n. *cysteinum* cysteine; L. v. *exigo* to demand; N.L. part. adj. *cysteinexigens* cysteine-demanding).

In addition to the characteristics that define the genus, cells are 1.2–1.8 μm long and 0.5–0.7 μm wide. Colonies on B-CYEα agar plates are flat, irregular, viscous and white to cream colour. Cysteine is required for growth. Subcultivation is successful on B-CYEα medium only when a large inoculum is used. Growth occurs at temperatures in the range 4–35 °C. Grows under aerobic and microaerobic conditions, but not anaerobic conditions. Negative for nitrate reduction, indole production, arginine dihydrolase, urease, β-glucosidase, gelatinase and β-galactosidase. Does not utilize glucose, arabinose, mannose, mannitol, *N*-acetyl-D-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate or phenylacetate. Positive for esterase (C4), leucine arylamidase and naphthol phosphohydrolase, but negative for alkaline phosphatase, esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The type strain is B1-EB^T (=JCM 30646^T=LMG 28693^T=NBRC 110909^T). Isolated from the fungus *Mortierella elongata* strain FMR23-6, which was isolated from cropland soil samples in Japan. The DNA G+C content of the type strain is 49.8 mol%.

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