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

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An endohyphal bacterium (strain B1-EBT) 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-EBT 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-EBT 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-EBT were C₁₆ : ₀, summed feature 3 (C₁₆ : ₁₀₇c and C₁₆ : ₁₀₆c) and summed feature 8 (C₁₈ : ₁₀₇c or C₁₈ : ₁₀₆c). 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-EBT 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-EBT (=JCM 30646T =LMG 28693T =NBRC 110909T).

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-Martínez et al., 2007).

In our previous study, bacteria living in association with the soil fungus Mortierella elongata were characterized...
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-oxoglutarate (B-CYEz) 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 × 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-CYEz 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-CYEz 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 (bio-Mériex) 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-CYEz agar plate medium at pH 6.9. To examine the strain’s cysteine requirement, B-CYEz agar medium (CM655 and SR110; Kanto Chemical) and B-CYEz 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 O2-absorbing and CO2-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-CYEz 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

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**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\textsuperscript{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 (ASDF) 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\textsuperscript{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\textsuperscript{T} was used as an outgroup.](attachment:phylogenetic_tree.png)
The colonies of strain B1-EB\textsuperscript{T} were flat, irregular, viscous and white to cream colour on B-CYE agar plates. Cells of strain B1-EB\textsuperscript{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\textsuperscript{T} on B-CYE medium were successful and visible growth was observed at 3 days of incubation only when a large inoculum was used. Strain B1-EB\textsuperscript{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-CYE agar plates. Growth of strain B1-EB\textsuperscript{T} was not observed on the B-CYE agar medium without L-cysteine after 7 days of incubation at 30 °C. The phylogram was visualized using FigTree v. 1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree/).

The major cellular fatty acids (>5.0 %) of three-day-cultured cells were C\textsubscript{16}:0 (23.2 %), summed feature 8 (C\textsubscript{18}:1\textsubscript{9}O7c or C\textsubscript{18}:1\textsubscript{10}O6c; 26.6 %), summed feature 3 (C\textsubscript{16}:1\textsubscript{10}O7c and C\textsubscript{16}:1\textsubscript{10}O6c; 23.7 %), C\textsubscript{16}:1 2-OH (8.5 %) and summed feature 2 (C\textsubscript{14}:0 3-OH and C\textsubscript{16}:1 iso I; 5.1 %), and other minor fatty acids (<5.0 %) were C\textsubscript{16}:0 3-OH (2.4 %), C\textsubscript{14}:1 iso I (2.3 %), C\textsubscript{18}:1 2-OH (2.2 %), C\textsubscript{16}:0 2-OH (2.0 %) and C\textsubscript{12}:0 (1.3 %). The principal polar lipids of strain B1-EB\textsuperscript{T} were phosphatidylethanolamine, phosphatidylglycerol, diphasphatidylglycerol, 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\textsuperscript{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 rDNA gene sequence alignment revealed that strain B1-EB\textsuperscript{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 rDNA 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\textsuperscript{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\textsuperscript{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. mukēs -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\textsubscript{16}:0 9c summed feature 8 (C\textsubscript{18}:1\textsubscript{9}O7c or C\textsubscript{18}:1\textsubscript{10}O6c) and summed feature 3 (C\textsubscript{16}:1\textsubscript{10}O7c and C\textsubscript{16}:1\textsubscript{10}O6c). The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphasphatidylglycerol, as well as unknown aminolipids and aminophospholipids. The predominant

### Table 1. Differential characteristics of strain B1-EB\textsuperscript{T} and genera in the family Burkholderiaceae

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>7</th>
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<tbody>
<tr>
<td>Motility</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Catalase</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<td>Growth at 41 °C</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>DNA G+C content</td>
<td>49.8</td>
<td>59.0–69.5</td>
<td>61.9–65.8</td>
<td>64.6–65.4</td>
<td>55–59</td>
<td>63–69</td>
<td>63.9–66.6</td>
<td>59 ± 12</td>
<td>59.8–65.0</td>
<td>40.3–49.4</td>
</tr>
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</table>

*Growth at 42 °C*.
quinoine 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* (cyst.e.in.ex’i.gens. N.L. n. cystei-num cysteine; L. v. exigo to demand; N.L. part. adj. cystei-nexigens 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-CYE agar plates are flat, irregular, viscous and white to cream colour. Cysteine is required for growth. Subcultivation is successful on B-CYE 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-galacosidase, 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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