RESEARCH NOTE

Blastobotrys illinoisensis, an Unrecorded Anamorphic Yeast Strain Isolated from the Gut of the Earthworm *Eisenia fetida*

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ABBREVIATION

The GenBank/EMBL/DDBJ accession numbers for the combined sequences of the D1/D2 region of the Large subunit (LSU) rRNA gene and the Internal transcribed spacer (ITS) region of strain E4 are MK246186 and MK246187, respectively. The MycoBank number for Blastobotrys *illinoisensis* is MB830626.

ABSTRACT

Strain E4, an unrecorded species of dimorphic fungi, was isolated from the gut of earthworms collected in Gyeonggi Province, South Korea. Nucleotide sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region revealed that this species is a member of the genus *Blastobotrys, Blastobotrys illinoisensis*. Strain E4 differed from its closest known species, *B. mokoenaii* and *B. malaysiensis,* by harboring 3-5 and 12-14 nucleotide substitutions in the D1/D2 and ITS regions, respectively. Phylogenetic analysis based on concatenated sequences of the D1/D2 region of the LSU rRNA gene and the ITS region also indicated that strain E4 belongs to the *Blastobotrys* clade and is distinct from other related species in the clade. The previously unreported isolate could be distinguished from closely related species by its inability to ferment carbon sources. To our knowledge, this is the first report on the isolation of *Blastobotrys* species from the gut of earthworms in Korea. The strain used was E4 (=KCTC 27831=JCM 33428).

Key words: *Blastobotrys* sp., D1/D2, Earthworm, ITS (internal transcribed spacer), Yeast

Insects are a remarkably diverse and abundant group of animals on earth [1]. Studies on the insect gut have received considerable attention and have colonized many different habitats [2]. In most insects, gut symbionts are essential for survival and development, suggesting the presence of a core microbiome. Terrestrial insects, such as the soil invertebrates (collembolans, earthworms, and nematodes), have been reported to harbor a rich microbiome and putative symbionts [3]. Earthworms are considered to be excellent bioindicators of the relative health of soil and possess several qualities that predispose them to use in monitoring terrestrial ecosystems worldwide [4]. Earthworms increase microbial activities by providing their gut mucus with energetic and readily metabolizable compounds [5] and considerable physicochemical conditions such as neutral pH, high moisture, and ideal temperature conditions [6]. However, earthworms
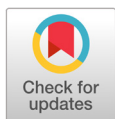
have poor enzymatic systems and rely on ingested soil microorganisms to degrade soil organic matter [7]. The earthworm gut is an effective tubular structure extending from the mouth to the anus; its different regions include the muscular pharynx, esophagus, intestine, and associated digestive glands. Gut contents usually comprise mucus, organic matter, and mineral matter. An analysis of gut contents in earthworms revealed the presence of different kinds of symbionts such as microfungi, bacteria, and protozoa; most microfungal species are present in the foregut, gradually decreasing in number in the mid and hindgut with the fewest in freshly laid casts [8]. Although the earthworm gut provides ideal conditions for growth of bacterial and fungal colonies [9], interactions between yeasts and earthworms are not well understood. In some associations, the fungus provides digestive enzymes and nutrition to the insect host.

The genus *Blastobotrys* was originally proposed as a hyphomycete by Von Klopotek [10]. Later, Kurtzman and Robnett [11,12] determined phylogenetic relationships among ascomycetous yeasts and yeast-like taxa based on the phylogenetic analysis of the D1/D2 region of the large subunit (LSU) rRNA gene and demonstrated that the *Blastobotrys* species are anamorphic members of Saccharomycetales, which are closely related to *Arxula* and *Sympodiomyces* and several species of *Candida*. In 2007, the relationships among species assigned to the ascosporic yeast clades, which include *Arxula*, *Blastobotrys*, *Sympodiomyces*, *Trichomonascus*, *Wickerhamiella*, and *Zygoascus*, were re-examined using phylogenetic analyses of multigene sequences, including the LSU rRNA gene, mitochondrial small subunit (SSU) rRNA gene, and cytochrome oxidase II. Based on multigene phylogenetic analysis, *Arxula*, *Blastobotry*, and *Sympodiomyces* belonged to the *Trichomonascus* clade, which is the teleomorphic state of the clade. Consequently, the anamorphic genera *Arxula*, *Blastobotrys*, and *Sympodiomyces,* are re-assigned as a single anamorphic genus, with *Blastobotrys* having taxonomic priority over *Sympodiomyces* and *Arxula* [13].

In a biodiversity study on the insect gut microbiome in Korea, unrecorded yeast strains belonging to *Rhodotorula taiwanensis*, *Dirkmeia churashimaensis*, *Moesziomyces aphidis*, *Moesziomyces antarcticus*, *Papiliotrema aspenensis,* and *Saitozyma flava* were isolated [14]. Based on DNA sequence comparisons, strain E4 isolated in Korea has been identified as an ascomycetous yeast species in the gut of the earthworm *Eisenia fetida*. Sequence analysis of the internal transcribed spacer (ITS) region and D1/D2 domains of the LSU rRNA gene identified this strain as *Blastobotrys illinoisensis*.

Eight earthworms and soil samples were collected from Gyeonggi-do province (37.4138°N, 127.5183° E), South Korea, and were transported in sterile beakers. Earthworms visually identified as *Eisenia fetida* were immediately washed with distilled water, frozen in liquid nitrogen, ground using a pestle and mortar, and stored at -80℃ until required. During dissection, frozen earthworms taken out from the freezer and immediately rinsed with 70% ethanol. The digestive tract of each earthworm was extracted by cutting the exoskeletons. The whole gut was stored in 1.5 mL microcentrifuge tubes, dissected, and cut into segments of 0.2-0.4 cm without surface sterilization. Between 30 and 40 tissue segments were then evenly placed in 9 cm diameter onto yeast extract-malt extract (YM) agar plates (pH 3.7-5.0; Difco) supplemented with 0.01% (w/v) chloramphenicol and then incubated at 25℃ in the dark. Colonies appeared after 3-5 days and were transferred to YM broth for propagation. Yeast strains were purified using YM agar and incubated at 25℃.

The plates were stored at 4℃, and re-streaking was performed for purification. Purified yeast strains were inoculated in YM broth supplemented with 10% glycerol (v/v) and stored at -80℃. For routine subculturing and maintenance, the strains were grown on YM agar or broth at 28℃.

Strain E4 was characterized morphologically, biochemically, and physiologically according to standard methods described by Kurtzman et al. [15]. Formation of pseudohyphae and true hyphae was investigated via slide culture on potato dextrose agar (PDA; 20% potato infusion, 2% glucose, and 1.5% agar) at 25℃ for 7 days. Ascospore formation was investigated by growing the strain on PDA, corn meal agar, 5% malt extract agar (5% malt extract and 1.5% agar), yeast extract-peptone glucose (YPD) agar (1% yeast extract, 2% peptone, 2% glucose, and 1.5% agar), and YM agar at 15℃ and 25℃ for 1 month. Growth at various temperatures was determined via cultivation on YM agar. Assimilation tests for carbon and nitrogen sources were performed using liquid media. Starved inocula were used in nitrogen and vitamin assimilation tests.

Genomic DNA was extracted and purified using the CTAB method [16]. The ITS (ITS1–5.8 S–ITS2) region of the rRNA gene was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by White et al. [17]. Amplification of the ITS region was performed using the following conditions: 95℃ for 3 min; followed by 37 cycles of 94℃ for 30 s, 52℃ for 30 s, and 72℃ for 30 s; and a final extension at 7℃ for 10 min. Meanwhile, the D1/D2 region of LSU rRNA was amplified with the primers NL1 (5′-GCATATCAATAAGCGGGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′), as described by Kurtzman and Robnett [12]. Amplification of the D1/D2 region was performed as follows: 94℃ for 6 min; followed by 40 cycles of 94℃ for 60 s, 50℃ for 60 s, and 72℃ for 60 s; and a final extension at 72℃ for 5 min. Sequence alignment was performed using CLUSTAL X 1.83 [18]. Reference sequences were retrieved from GenBank (accession numbers indicated in the phylogenetic tree). Phylogenetic and molecular evolutionary analyses using neighbor-joining [19] and maximum-likelihood methods were conducted in MEGA version 7 [20], and evolutionary distances were calculated using the general time-reversible (GTR) model [21]. Confidence levels of the clades were estimated through bootstrap analysis (1,000 replicates) [22], and only values above 50% were recorded for the resulting trees. *Schizosaccharomyces pombe* NRRL Y-12796[T] was used as an outgroup.

SPECIES DELINEATION AND PHYLOGENETIC PLACEMENT

A total of 10 yeast strains were isolated from the gut of earthworms during an investigation of earthworm gut-associated microbial communities in South Korea. Sequence analyses of the D1/D2 domains of the LSU rRNA gene revealed that E4 isolate belonged to *Blastobotrys illinoisensis*. Based on the pairwise sequence similarity of D1/D2 domains, the closest relatives of the species were *B. mokoenaii* CBS 8435[T] (99.4%) and *B. malaysiensis* CBS 10336[T] (99.2%). The D1/D2 sequences of the unreported species showed a sequence divergence of 0.5% (three nucleotide substitutions and zero gaps over 633 bases), 0.6% (four nucleotide substitutions and no gaps), and 0.8% (five nucleotide substitutions and zero gaps) from the closest relatives, *Blastobotrys illinoisensis*, *B. mokoenaii*, and *B. malaysiensis*, respectively. Results of the pairwise sequence similarity based on ITS regions revealed that the closest relatives of the strain

were *B. mokoenaii* CBS 8435^T (95.7%) and *B. malaysiensis* CBS 10336^T (96.6%). A comparison of the ITS sequences revealed that the novel species showed a sequence divergence of 2.5% (14 nucleotide substitutions and 7 gaps over 566 bases), 2.2% (12 nucleotide substitutions and 11 gaps over 539 bases), and 2.1% (12 nucleotide substitutions and 7 gaps over 548 bases) from its closest relatives *B. mokoenaii* and *B. malaysiensis*. Furthermore, according to the DNA barcoding analysis of yeast species by Vu et al. [23], ITS and LSU can be used as taxonomic thresholds (96.3 and 99.5%, respectively) to discriminate yeast species. Therefore, we concluded that the novel isolates belonged to *Blastobotrys illinoisensis*.

A phylogenetic tree was constructed using the neighbor-joining method based on the D1/D2 LSU rRNA gene sequences of the unreported species, its closest relatives, and members of the *Blastobotrys* clade, as defined by Kurtzman et al. [15]. The unreported species formed a clade with *B. illinoisensis* CBS 10339^T (Fig. 1). The phylogenetic relationships of the unreported species and its closely related species in the subclade were also supported by maximum likelihood analysis (data not shown). *Schizosaccharomyces pombe* NRRL Y-12796^T was used as an outgroup.
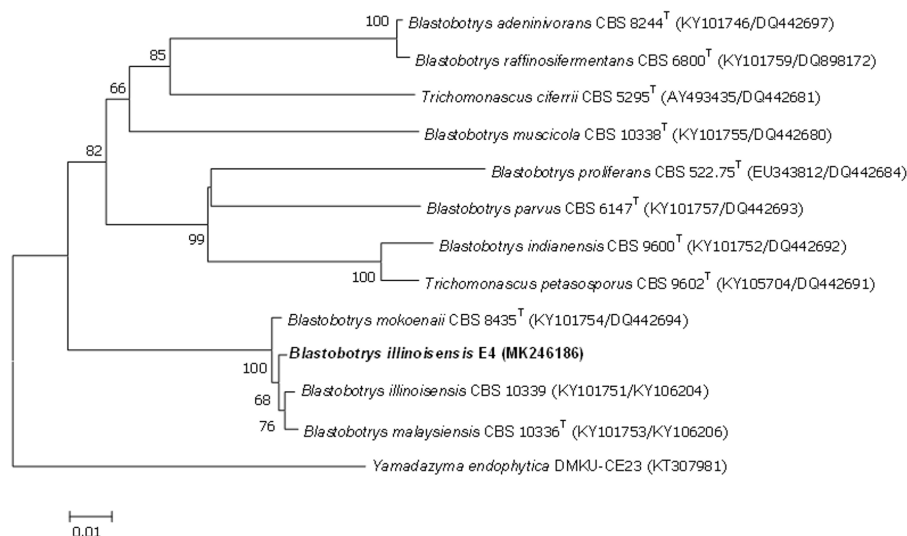
Fig. 1. Phylogenetic placement of *Blastobotrys illinoisensis* E4 and related species using the neighbor-joining method and maximum-likelihood analysis of concatenated sequences of the internal transcribed spacer (ITS) region and the D1/D2 region of the large subunit (LSU) rRNA gene. *Yamadazyma endophytica* DMKU-CE23 was used as the out-group species. Bootstrap values based on 1,000 replications are shown at the branch nodes. Bar, 0.01 substitutions per nucleotide position.

Collectively, sequence data and phylogenetic analysis of the D1/D2 region of the LSU rRNA (Fig. 2) and the ITS region suggested that the isolate is a novel species of the genus *Blastobotrys*. Moreover, the ITS region sequence was not available for many *Blastobotrys* species, except for *B. illinoisensis* CBS 10339^T (DQ898169), *B. malaysiensis* CBS 10336^T (DQ898170), *B. raffinosifermentans* CBS 6800^T (DQ898172), *B. adeninivorans* CBS 8244^T (DQ898168), and *B. mokoenaii* CBS 8435^T (DQ89817).
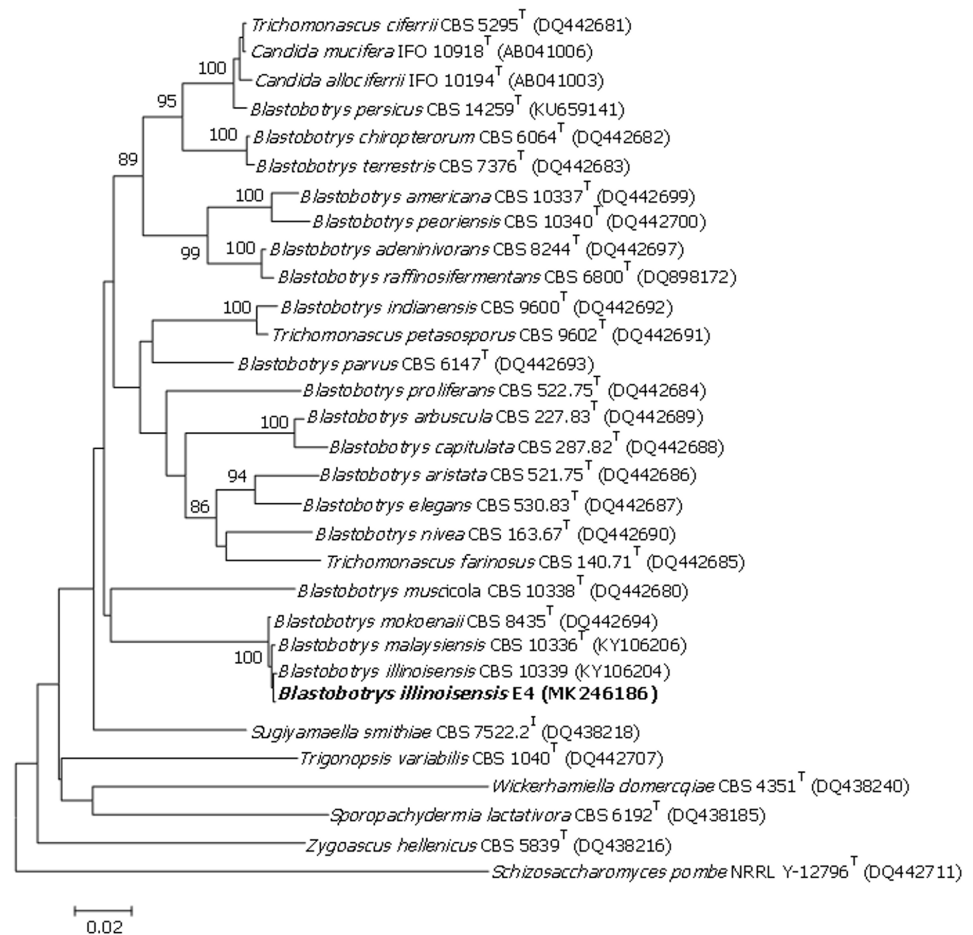
Fig. 2. Phylogenetic placement of *Blastobotrys illinoisensis* E4 and related species using the neighbor-joining method and maximum-likelihood analysis of D1/D2 large subunit (LSU) rRNA gene sequences. *Schizosaccharomyces pombe* was used as the out-group species. Bootstrap values based on 1,000 replications are shown at the branch nodes. Bar, 0.02 substitutions per nucleotide position.

Cells of strain E4 were butyrous, and colonies appeared white and relatively raised. Detailed morphology is provided in the succeeding section. The morphological and physiological characteristics of strain E4 were similar to those of *Blastobotrys spp.* [24]. Analysis of its sporulation activity revealed the absence of conjugation and formation of asci or ascospores. Budding cells were formed via multilateral budding, and formation of true hyphae was observed (Fig. 3). A similar type of budding has been reported in the closest species *Blastobotrys persicus*; however, this species lack true hyphae [24]. The detailed phenotypic characteristics of strain E4 and related *Blastobotrys* spp. are presented in Table 1 and Table 2. In addition, *B. koreensis* assimilates trehalose and melezitose (weak) and do not assimilate D-arabinose, raffinose, and ethanol, compared to *B. illinoisensis*. Strain E4 did not ferment carbon sources, did not grow in 10% NaCl containing 5% glucose, and had different assimilation profiles compared to *Blastobotrys illinoisensis*, *B. mokoenaii*, and *B. malaysiensis* (Table 1). Furthermore, strain E4 can be differentiated from the closest relative species based on phenotypic and chemotaxonomic characteristics, and thus the novel species was assigned to the genus *Blastobotrys*.

Table 1. Phenotypic characteristics of *Blastobotrys illinoisensis* E4 and its closely related species

Characteristics	Species[a]			
	1	2	3	4
Fermentation				
Glucose	-	+	+	+
Galactose	-	+	+	+
Sucrose	-	w	W	-
Maltose	-	+	+	-
Assimilation of carbon compounds				
L-Sorbose	+	+	+	+
D-Ribose	-	-	+	-
D-Xylose	+	+	+	+
L-Arabinose	+	+	+	+
D-Arabinose	-	+	+	-
L-Rhamnose	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	-	+	ND
Maltose	+	+	+	+
Methyl α-D-glucoside	+	+	+	+
Salicin	+	+	+	-
Melibiose	-	-	-	-
Lactose	-	-	+	-
Raffinose	-	+	+	-
Meleizitose	w	-	W	-
Erytritol	+	+	+	+
Ribitol	+	+	+	+
D-Glucitol	+	+	+	+
D-Gluconate	+	+	-	W
Ethanol	-	+	+	+
Growth with 10% NaCl+5% glucose	-	+	+	+

+: growth reactions positive; -: growth reactions negative; w: weakly positive; ND: no data.

[a]Species: 1, *Blastobotrys illinoisensis* E4; 2, *B. illinoisensis* CBS 10339[T]; 3, *B. mokoenai* CBS 8435[T]; 4, *B. malaysiensis* CBS 10336[T]. Data for species 1 are obtained from the present study, and those for species 2, 3, and 4 are obtained from a previous study [13].

Table 2. Morphological characteristics of *Blastobotrys illinoisensis* E4 and its closely related species

| Characteristics	Species[a]			
	1	2	3	4
Cell shape and size	Oval shape 3-4 μm	Spherical 2.0-4.0 μm	Ovoid 2.8-4.1μm	Spherical 3.0-5.5 μm
Spore formation	Blastoconidia	Spherical endospores in hyphal cells	No spores	No ascospores
Hyphae formation	True hyphae	Pseudohyphae and true hyphae	Pseudohyphae	True hyphae
Budding	Multilateral	Multilateral	Multilateral	Multilateral
Colony morphology	White colored, butyrous, glistening, entire margin	Powdery appearance and a prominent mycelial fringe	Cream colored, raised, butyrous, matted, rhizoid	tannish-white, dull, somewhat butyrous and with a prominent mycelial fringe
pH	5-7	ND	7-10	ND
Temperature	28℃	25℃	25℃	25℃
Growth media	YMA agar	5% ME agar	Glucose-peptone-yeast extract agar	5% ME agar

ND: No Data; YMA: Yeast Malt agar; ME: Malt Extract.

[a]Species: 1, *Blastobotrys illinoisensis* E4; 2, *B. illinoisensis* CBS 10339[T]; 3, *B. mokoenai* CBS 8435[T]; 4, *B. malaysiensis* CBS 10336[T]. Data for species 1 are obtained from the present study, and those for species 2, 3, and 4 are obtained from a previous study [13].

Description of *Blastobotrys illinoisensis* E4

Growth on yeast malt agar (YMA): After incubation for 72 h at 28°C, colonies appear white, butyrous, glistening, and with entire margins (Fig. 3). Cells are spheroidal to ovoidal (3-4×2.5-4 μm), and occur singly, in pairs, or in small clusters. Budding is multilateral (Fig. 2b). True hyphae are observed on YMA and PDA agar. Ascus formation is absent. Pseudohyphae are rare on yeast extract peptone glycerol (YPG) media, PDA, and cornmeal agar. Protuberances, an indication of potential early stage of germ tube production, and short chains of blastoconidia (Fig. 3) are formed. Colonies grow at 15°C and 37°C, but not at 40°C.
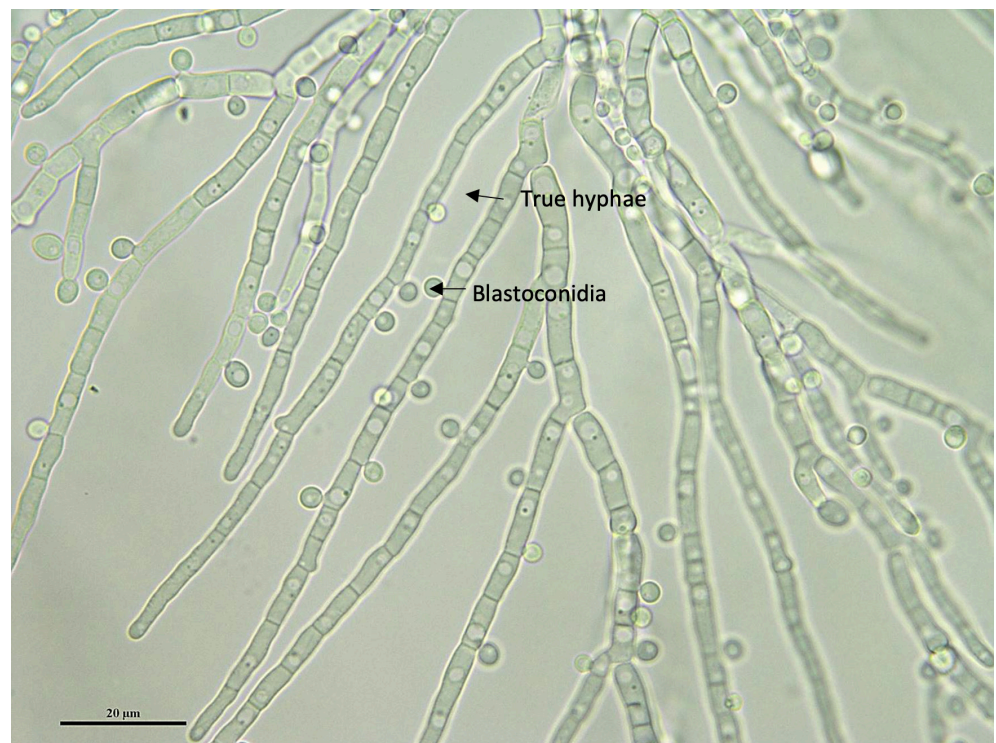
Fig. 3. Morphology of *Blastobotrys illinoisensis* E4 cells on yeast malt (YM) agar after 7 days at 25　, true hyphae and blastoconidia. Scale bar, 20 μm..

Fermentation is absent. *N*-acetyl glucosamine, L-arabinose, D-galactose, D-glucose, L-sorbose, D-xylose, L-rhamnose, sucrose, trehalose, maltose, methyl α-D-glucoside, cellobiose, salicin, erythritol, ribitol, D-glucitol, D-mannitol, *myo*-inositol, D-glucono-1,5-lactone, D-gluconate, D-glucuronate (weak), meleizitose (weak), D-galacturonic acid, glycerol (weak), DL-lactate (weak), succinate, citrate, and xylitol are assimilated. No growth was observed when D-ribose, melibiose, lactose, raffinose, methanol, and ethanol are used. Ammonium sulfate, l-lysine, and cadaverine are assimilated as nitrogen sources. Potassium nitrate, sodium nitrate, ethylamine HCL, and creatine are not assimilated. Growth on 50% and 60% glucose-yeast extract agar is observed, and growth on 0.01 and 0.1% cycloheximide is not observed. No growth was observed in 10% NaCl with 5% glucose. Acid and starch formation tests are negative. Diazonium Blue B (DBB) reaction and urease are negative.

Strain E4 was isolated from the gut of *Eisenia fetida* collected from Onam-si, Korea. The strain is preserved in a metabolically inactive state at the Korean Collection for Type Cultures (KCTC), Republic of Korea (KCTC 27831) and the Japan Collection of Microorganisms, Japan (JCM 33428). The MycoBank accession number is MB830626.

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CONFLICT OF INTEREST

All authors certify that there are no conflicts of interest with any financial organization regarding the material discussed in the manuscript.

ETHICAL APPROVAL

This article does not contain any studies involving human participants or animals performed by any of the authors.

AUTHOR'S CONTRIBUTIONS

All authors equally contributed to this work.

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