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Isolation and characterization of aerobic microorganisms with cellulolytic activity in the gut of endogeic earthworms

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Summary. The ability of earthworms to decompose lignocellulose involves the assistance of microorganisms in their digestive system. While many studies have revealed a diverse microbiota in the earthworm gut, including aerobic and anaerobic microorganisms, it remains unclear which of these species contribute to lignocellulose digestion. In this study, aerobic microorganisms with cellulolytic activity isolated from the gut of two endogeic earthworms, *Amynthas heteropoda* (Megascolecidae) and *Eisenia fetida* (Lumbricidae) were isolated by solid culture of gut homogenates using filter paper as a carbon source. A total of 48 strains, including four bacterial and four fungal genera, were isolated from two earthworm species. Characterization of these strains using enzyme assays showed that the most representative ones had exocellulase and xylanase activities, while some had weak laccase activity. These findings suggest that earthworms digest lignocellulose by exploiting microbial exocellulase and xylanase besides their own endocellulase. Phylogenetic analysis showed that among the cellulolytic isolates in both earthworm species *Burkholderia* and *Chaetomium* were the dominant bacterial and fungal members. [**Int Microbiol** 2012; 15(3):121-130]

Keywords: *Burkholderia* · *Chaetomium* earthworms · lignocellulose digestion · cellulases · xylanases

Introduction

Earthworms are well known for their contribution to lignocellulose decomposition in soil. However, it has long been recognized that most earthworms and other animals living in soil do not produce their own endogenous cellulase, instead depending on cellulase from their resident gut microorganisms. However, genes encoding endogenous cellulase in several insects have been recently isolated [37], such as the endogenous endocellulase gene of the earthworm *Pheretima hilgendorf* (Megascolecidae), first isolated in 2009 [26]. Despite these newly discovered abilities, earthworms cannot assimilate lignocellulose by means of endocellulase alone, since efficient lignocellulose degradation requires the synergistic action of a suite of other enzymes, including exocellulase, hemicellulase (e.g., xylanase), and lignin peroxidase [23]. According to current views, a synergistic earthworm–microbial digestive system (dual-digestive system) is indispensable for the digestion and utilization of lignocellulose by earthworms [5].

Since the earthworm gut is free of detectable oxygen [18], it would appear that lignocellulose digestion is carried out mainly by anaerobic microorganisms in the gut. However, cellulolytic anaerobes have yet to be isolated from

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the earthworm gut. In contrast, several studies have demonstrated that the earthworm gut contains an abundance of aerobic microorganisms (aerobes) in amounts nearly equivalent to that of anaerobes [9,19]. Moreover, some aerobes have been shown to proliferate during passage through the earthworm gut, reaching densities greater than in soil [12,21,27]. Considering the dual-digestive system described above and the abundance of aerobes in the earthworm gut, we hypothesized that some species of cellulolytic aerobes can survive and contribute to lignocellulose digestion in the gut. Although several studies based on culture-dependent methods have found that most aerobes in the gut microbiota belong to genera found also in soil [1,7,27], their contribution to lignocellulose digestion in the gut remains unclear, except in the case of *Cellulomonas* sp., the sole cellulolytic aerobe isolated so far from the gut [8]. Several metagenome studies have comprehensively revealed diverse bacterial biota in the earthworm gut [3,12,25,33], but so far these studies have been unable to detect cellulolytic strains, the exception being the report by Beloqui et al. [3] describing the detection of DNA clones for *Cellovibrio* in the earthworm gut. Moreover, knowledge of fungal members in the gut is lacking in most metagenome studies. Therefore, the question of which species of cellulolytic aerobes contribute to lignocellulose digestion in the earthworm gut remains to be fully answered.

In this study, cellulolytic aerobes in the gut of two endogeic earthworm species, *Amynthas heteropoda* (Megascolecidae) and *Eisenia fetida* (Lumbricidae), were isolated and characterized. Our findings are described herein.

Materials and methods

Chemicals. Cellulose (Avicel), carboxymethyl cellulose (CMC), birchwood xylan, and dinitrosalicylic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Reagents for the molecular biological analyses, including Taq DNA polymerase, were from Takara Bio (Kyoto, Japan). All other chemicals were from Wako Pure Chemicals (Kyoto, Japan).

Preparation of culture media. Cellulolytic microorganisms were isolated using a mineral salts agar medium (pH 5.3) with a Whatman no. 7 filter paper, as the sole carbon source, placed on the surface of the agar. The composition of the medium was described previously [14].

Collection of earthworms. *Amynthas heteropoda* specimens were collected from farmland at the Experimental Agriculture Station of Yamaguchi University, Yamaguchi, Japan. *Eisenia fetida* specimens were purchased from a commercial supplier (Marunichi, Fukushima, Japan), where they had been reared for vermicompost. The earthworms were washed with autoclaved tap water, their body surface was sterilized by a brief rinse with 70 % ethanol, and immediately anesthetized on crushed ice.

Cultivation and isolation of gut microorganisms. Whole-intestine sections (including foregut, midgut, and hindgut) of the earthworm (20 worms for each species) were dissected out and homogenized in autoclaved distilled water containing 0.5-mm glass beads, with vortex mixing for 5 min with a vortex mixer. The resulting suspension was serially diluted with water and used as inoculum. For determination of the number of colony-forming units (CFU) of total culturable gut microorganisms, 1 ml of diluted suspension was inoculated with mineral salts agar containing 1 % glucose (glucose-agar). Cellulolytic microorganisms were isolated in mineral salts agar medium (pH 5.3) with a Whatman no. 7 filter paper, as the sole carbon source, placed on the surface of the agar. The agar was then overlaid with another Whatman no. 7 filter paper (70 mm in diameter) to sandwich the suspension between the agar surface and filter paper. The agar plates were incubated statically in the dark at 30 °C for 2 weeks under oxic conditions. Emerging colonies on the filter paper were counted, and unique isolates, as determined by morphology, were purified three times on fresh YM agar (10 g glucose / l, 3 g yeast extract / l of, 3 g malt extract / l , 5 g peptone / l and 20 g Bacto agar / l).

Phylogenetic study of isolates. Cell mycelia of fungal isolates were obtained from 20-ml pure cultures in YM broth (10 g glucose/l, 3 g yeast extract/l, 3 g malt extract / l, and 5 g peptone/l). The cell pellets of the bacterial isolates were harvested from 1-ml aliquots of pure cultures. DNA extraction from the isolates, PCR amplification of internal-transcribed spacer (ITS) regions (approximately 450 bp, including ITS1, 5.8S, and ITS2 regions) and partial 16S ribosomal DNA (approximately 420 bp), and direct sequencing of the amplified DNA fragments were carried out as described by Fujii et al. [14]. The similarities of the obtained sequences with known species were determined by comparison with sequence data in the GenBank, EMBL, and DDBJ databases using the BLAST algorithm [2]. Phylogenetic trees were constructed by the neighbor-joining method contained in the Clustal W program [30,34]. The ITS region DNA and 16S rDNA sequences for the isolates were deposited in the DDBJ database under the accession numbers shown in Table 1.

Enzyme assays. Selected isolates were cultivated in 20 ml of Mandels and Weber medium for 1 week at 30 °C with shaking at 150 rpm [24]. The medium contained: 1.4 g $(NH_4)_2SO_4$/l; 0.3 g $(NH_2)_2CO$/l; 2.0 g KH_2PO_4/l; 0.3 g $MgSO_4 \cdot 7H_2O$/l; 0.4 g $CaCl_2 2 \cdot H_2O$/l; 5.0 mg $FeSO_4 \cdot 7H_2O$/l; 2.2 mg $MnSO_4 \cdot 5H_2O$/l; 1.4 mg $ZnSO_4 \cdot 7H_2O$/l; 3.7 mg $CoCl_2 \cdot 6H_2O$/l; 0.75 g peptone/l; and 0.25 g yeast extract/l. The culture supernatants (20 ml) of the various isolates were separated from the microbial biomass and insoluble materials by centrifugation at 3000 ×*g* for 10 min, followed by filtration through a Vivaspin-20 concentrator (GE Healthcare, Little Chalfont, UK). The residue on the filter membrane was suspended in 1 ml of 50 mM citrate buffer (pH 4.8) and used as the enzyme solution. The protein concentration of the enzyme solution was determined by the method of Bradford [4]. Exocellulase, endocellulase, and xylanase activities were measured using Avicel, CMC, and birchwood xylan as substrates, respectively, according to the DNS method [17]. Laccase activity was determined using syringaldazine as substrate, following the method of Leonowicz and Grywnowicz [22].

Results

CFU counts of culturable gut microorganisms and isolation of cellulolytic microorganisms. The number of microorganisms that could be cultured by plating on glucose-agar were 2.8×10^7 CFU $(g\text{-gut})^{-1}$ and 1.5×10^7 CFU $(g\text{-gut})^{-1}$ for *A. heteropoda* and *E. fetida*, respectively.

Table 1. Accession numbers for 16S rDNA (bacterial isolates) and ITS region DNA (fungal isolates) sequences

Strain	Accession no.	Strain	Accession no.
Amy-2	AB728507	Eis-3	AB728520
Amy-3	AB728535	Eis-4	AB728521
Amy-4	AB728536	Eis-5	AB728522
Amy-5	AB728537	Eis-6	AB728523
Amy-6	AB728508	Eis-7	AB728546
Amy-8	AB728538	Eis-8	AB728524
Amy-9	AB728539	Eis-10	AB728525
Amy-11	AB728509	Eis-11	AB728526
Amy-12	AB728510	Eis-14	AB728527
Amy-13	AB728540	Eis-15	AB728528
Amy-14	AB728511	Eis-16	AB728547
Amy-15	AB728512	Eis-17	AB728548
Amy-16	AB728513	Eis-18	AB728529
Amy-18	AB728541	Eis-19	AB728549
Amy-19	AB728514	Eis-20	AB728550
Amy-20	AB728515	Eis-21	AB728551
Amy-21	AB728516	Eis-22	AB728552
Amy-22	AB728542	Eis-23	AB728530
Amy-23	AB728517	Eis-24	AB728531
Amy-24	AB728518	Eis-26	AB728532
Amy-25	AB728519	Eis-27	AB728533
Amy-26	AB728543	Eis-28	AB728534
Eis-1	AB728544	Eis-29	AB728553
Eis-2	AB728545	Eis-30	AB728554

After a 2-week cultivation, microbial colonies with various morphologies emerged on the filter paper, and the concentrations of microorganisms were 8.5×10^3 CFU $(\text{g-gut})^{-1}$ and 2.1×10^4 CFU $(\text{g-gut})^{-1}$ for *A. heteropoda* and *E. fetida*, respectively. Thus the cellulolytic strains accounted for approximately 0.03 % (*A. heteropoda*) and 0.14 % (*E. fetida*) of the total culturable aerobes. From the *A. heteropoda* gut, 26 isolates (strains Amy-1 to 26) were obtained based on colony morphology, among which 22 isolates (13 bacterial and 9 fungal) were able to grow through repeated subculture, while four isolates (Amy-1, 7, 10, and 17) failed to grow upon subculturing for unknown reasons. From the *E. fetida* gut, 30 isolates (strains Eis-1 to 30) were obtained, among which 26 isolates (15 bacterial and 11 fungal) were successfully subcultured, while four isolates (Eis-9, 12, 13, and 25) became extinct during subculturing.

Attempts were also made to isolate cellulolytic microorganisms from the gut of earthworms that had been kept on sterile sands for 5 days. However, no microbial colonies emerged on the filter paper.

Table 2. Enzyme activities of representative cellulolytic isolates

		Exocellulase*		Endocellulase*		Xylanase*		Laccase**	
Strain	Genus	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Amy-2	*Burkholderia*	0.009	0.008	ND		0.101	0.077	ND	
Amy-3	*Chaetomium*	0.009	0.003	0.009	0.003	ND		ND	
Amy-4	*Staphylotrichum*	0.008	0.005	0.036	0.032	0.089	0.044	ND	
Amy-5	*Penicillium*	0.018	0.000	1.151	1.020	0.519	0.373	ND	
Amy-9	*Fusarium*	0.024	0.021	1.125	0.978	2.773	1.883	0.002	0.001
Amy-11	*Burkholderia*	0.010	0.005	ND		0.106	0.096	ND	
Amy-13	*Chaetomium*	0.007	0.003	0.024	0.011	0.109	0.040	ND	
Amy-18	*Fusarium*	0.018	0.016	0.312	0.286	2.322	0.988	0.003	0.001
Amy-21	*Burkholderia*	0.012	0.006	0.012	0.007	0.135	0.119	ND	
Amy-23	*Burkholderia*	0.008	0.006	ND		0.046	0.026	ND	
Amy-24	*Burkholderia*	0.009	0.003	ND		0.100	0.051	ND	
Amy-26	*Chaetomium*	0.007	0.002	0.044	0.023	0.027	0.019	ND	
Eis-1	*Chaetomium*	0.008	0.003	ND		0.020	0.018	ND	
Eis-3	*Pseudomonas*	0.005	0.003	ND		0.094	0.064	ND	
Eis-4	*Herbaspirillum*	0.005	0.004	0.032	0.017	0.181	0.130	0.005	0.002
Eis-5	*Burkholderia*	ND		0.018	0.035	0.176	0.081	ND	
Eis-7	*Staphylotrichum*	0.016	0.010	0.183	0.032	1.834	1.307	0.009	0.004
Eis-8	*Burkholderia*	ND		0.009	0.008	0.089	0.076	ND	
Eis-10	*Burkholderia*	0.008	0.006	ND		0.135	0.117	ND	
Eis-15	*Enterobacter*	ND		0.005	0.002	0.038	0.021	ND	
Eis-18	*Burkholderia*	ND		0.009	0.008	0.088	0.031	ND	
Eis-19	*Chaetomium*	0.012	0.011	ND		0.156	0.115	ND	
Eis-20	*Chaetomium*	0.010	0.008	0.032	0.017	0.109	0.090	ND	
Eis-21	*Chaetomium*	0.006	0.004	0.006	0.005	0.179	0.088	ND	
Eis-23	*Burkholderia*	0.008	0.006	0.053	0.024	0.054	0.037	ND	

The values shown are the mean ± standard deviation of independent experiments performed in triplicate.
*Enzyme activity was expressed as U min^{-1} $(mg\text{-}protein)^{-1}$. One unit corresponds to 1 μmol reducing sugar equivalent produced during the enzyme reaction [17].
**Enzyme activity was expressed as U s^{-1} $(mg\text{-}protein)^{-1}$. One unit corresponds to 1 μmol syringaldazine oxidized during the enzyme reaction [22].
SD, standard deviation. ND, not detected, i.e., < 0.001 U $(mg\text{-}protein)^{-1}$.

Phylogenetic analysis of cellulolytic isolates. Figure 1A and 1B shows the phylogenetic trees for the bacterial and fungal isolates, as constructed using the neighbor-joining method. For bacterial isolates, *Burkholderia* spp. were dominant in the cellulolytic bacterial biota in both earthworm species, and *E. fetida* additionally contained sev-

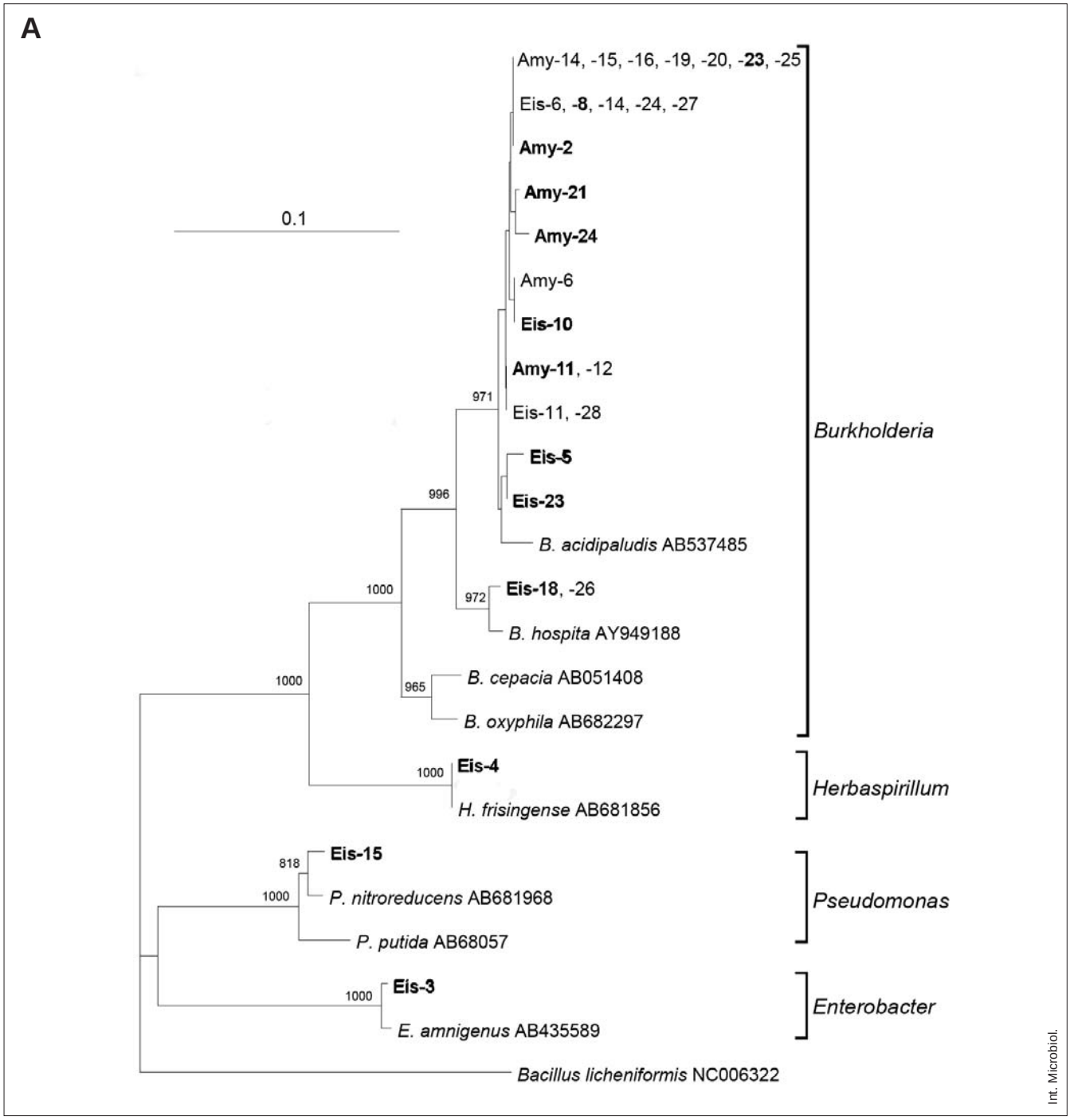

Fig. 1A. Phylogenetic tree of cellulolytic isolates and related species constructed using the neighbor-joining method. Trees for bacterial isolates are based on partial 16S rDNA sequences. The scale bar represents 0.1 base substitutions per nucleotide.

eral strains belonging to other genera (*Herbaspirillum*, *Enterobacter*, and *Pseudomonas*). The cellulolytic fungal biota was composed mainly of *Chaetomium* members, while *A. heteropoda* contained additional fungal genera (*Penicillium*, *Fusarium*, and *Staphylotrichum*).

Enzyme activity of the isolates. Twenty-five isolates (13 bacterial and 12 fungal) were selected as representative strains (indicated in bold letters in Fig. 1A and 1B) based on their phylogenetic positions and subsequently examined for cellulase, xylanase, and laccase activities.

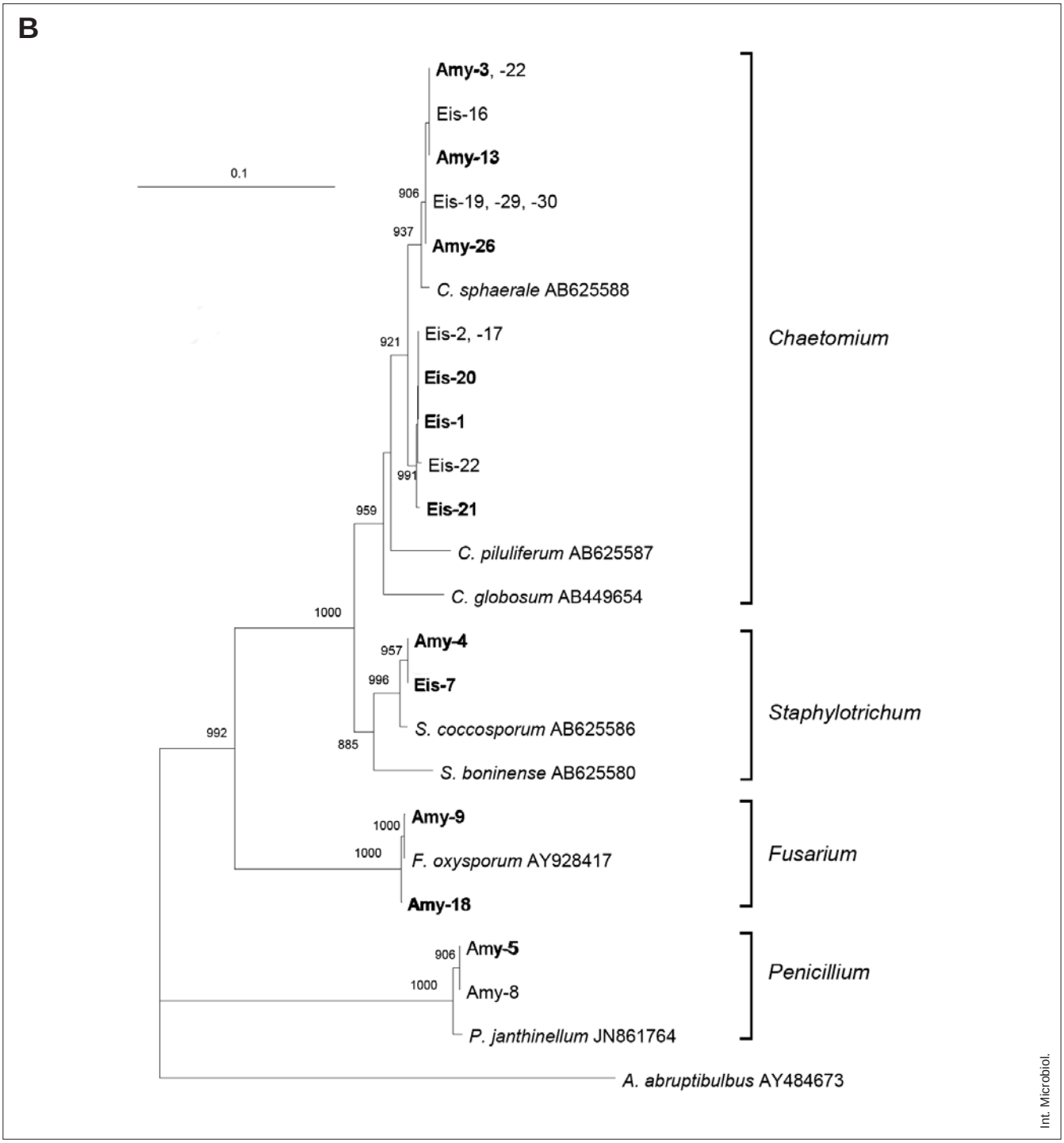

Fig. 1B. Phylogenetic trees of cellulolytic isolates and related species constructed using the neighbor-joining method. Trees for fungal isolates are based on ITS region sequences. Accession numbers for the isolates are shown in Table 1. The scale bar represents 0.1 base substitutions per nucleotide. Bootstrap values [11] above 50 % (of 1000 samplings) are shown at the internodes. The strains indicated in bold letters were assayed for cellulase, xylanase, and laccase activities.

Table 2 summarizes the enzyme activities in the culture supernatants of each examined isolate. Notably, exocellulase activity was detected in the culture supernatants of most tested strains, with relatively greater activity in several fungal strains (Amy-5, -9, 18, and Eis-7). A number of isolates also showed endocellulase and xylanase activities, especially

strains Amy-5, -9, and -18. Additionally, some strains (Amy -9, -18, and Eis-4, -7) had weak laccase activity.

Discussion

Earthworms can be viewed as ecological engineers that contribute to the digestion of lignocellulose. However, endogenous cellulase alone cannot accomplish lignocellulose digestion; rather, a suite of additional microbial enzymes are needed. Metagenome studies have begun to shed light on microbial ecology, especially regarding the bacterial biota, in the earthworm gut. However, metagenomic approaches are unsuitable for identifying cellulolytic species in the biota because barcode genes (e.g., 16S-rDNA) are mainly used in the analysis. Moreover, most metagenome studies lack knowledge of fungal members. Therefore, culture-dependent studies are still useful to identify cellulolytic organisms in the gut microbiota. While there are many reports on the isolation of cellulose degraders in the gut of insects such as termites [36], few studies have isolated or identified their counterparts in the earthworm gut [3,8]. In the present work, cellulolytic aerobes belonging to various genera were isolated and characterized. To the best of our knowledge, our study is the first to determine the activities of the cellulases, xylanases, and lignin peroxidases of cellulolytic isolates obtained from the earthworm gut. The dominant bacterial and fungal species among the isolates were *Burkholderia* spp. and *Chaetomium* spp., respectively, in both earthworm species. Since the two earthworm species were of different origins, it can be concluded that the contribution of cellulolytic aerobes to lignocellulose digestion in the earthworm gut is not host-specific, but common among endogeic earthworms. Interestingly, in earthworms that had been placed on sterile sands for 5 days, cellulolytic microorganisms could not be isolated from the worms' gut, suggesting that the isolates are not symbionts of earthworms but are derived from ingested foods, such as litter fragments. While *Burkholderia* and *Chaetomium* are known as soil microorganisms with cellulolytic activities [10,14–16], the isolation of both genera from an insect gut has not been previously reported. Hence this is the first description of their contribution to lignocellulose digestion in the earthworm gut.

Efficient lignocellulose digestion in the earthworm gut requires the synergistic action of a suite of enzymes, including exocellulase, hemicellulase (e.g., xylanase), and lignin peroxidase, as well as endocellulase [23]. Endogenous cellulase genes have been identified in various insects, including earthworms, and all of them encode "endocellulases" [37]. Ueda et al. [35] recently purified the cellulolytic multienzyme complex of *E. fetida* and analyzed its enzyme activity. They found that the complex has CMCase (endocellulase) and xylanase but not avicelase (exocellulase) activity, suggesting that the earthworm cannot produce exocellulase. In contrast, almost all representative isolates in this study were found to have "exocellulase" activity. The cellulose fiber in lignocellulose contains both crystalline and amorphous components that can be depolymerized by exocellulase and endocellulase, respectively [23]. Our results suggest that earthworms digest the amorphous part of cellulose by using endogenous endocellulase but depend on microbial exocellulase to digest the crystalline part of cellulose. Additionally, many isolates also showed xylanolytic activity, and some showed laccase activity, suggesting that microbial xylanase and lignin peroxidase play important roles in removing xylan and lignin and in exposing cellulose fibers on the lignocellulose surface.

The presence of cellulolytic aerobes in the earthworm gut able to degrade lignocellulose is surprising, since the earthworm gut is free of oxygen and anaerobic bacteria that ferment low molecular-weight sugars (e.g., glucose, cellobiose) to organic acids comprise a major population in the gut microbiota [19,39]. However, the proliferation of aerobic microorganisms in the earthworm gut has been reported in several studies [9,19,21,27,28,32,38]. These discrepant results can be explained by the following hypothesis (Fig. 2). Aerobic soil microorganisms, including cellulolytic ones, associated with lignocellulose are ingested by an earthworm and then introduced into the anterior digestive tract (pharynx or esophagus), where the moisture-rich and oxic conditions allow their growth and the production of enzymes (e.g., cellulase and xylanase) for lignocellulose digestion (Fig. 2A). Subsequently, the microorganisms are exposed to the anoxic conditions of the gizzard and intestine [39], resulting in the inactivation of aerobic microorganisms but the continued activity of their enzymes, including those that saccharify lignocellulose during gut passage (Fig. 2B).

The resulting degradation products (e.g., glucose, cellobiose, xylose, and their oligosaccharides) are thus consumed by the earthworm but also used by anaerobes as a fermentation substrate [19], as shown in Fig. 2B. Lastly, the grown biomass of anaerobes is digested and consumed by the earthworm as a source of essential amino acids and fatty acids [29,31], as shown in Fig. 2C. Glycosidase activity should remain even if the growth of enzyme-producing microorganisms is halted by anaerobiosis, because these

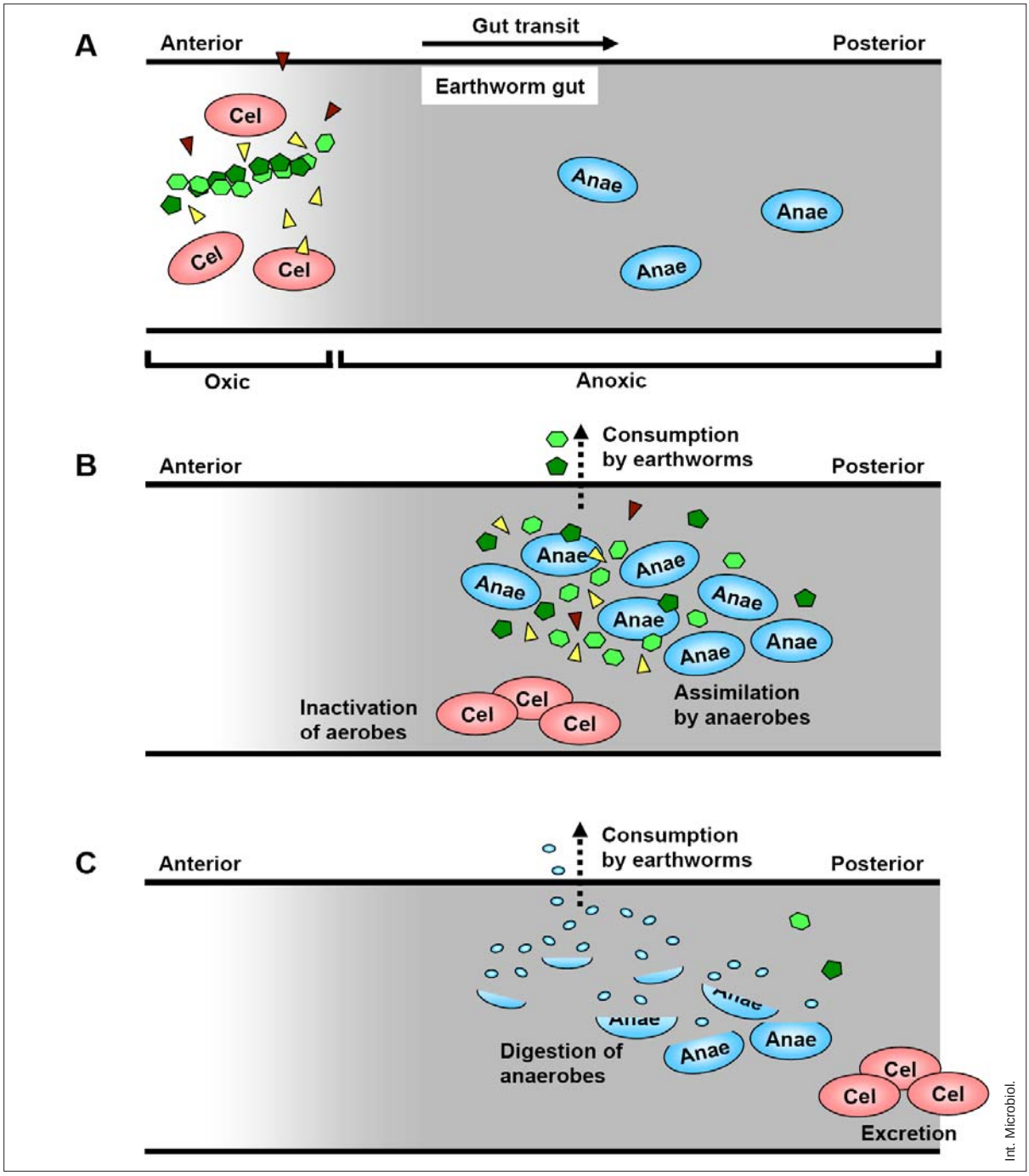

Fig. 2. Proposed hypothesis for the role of cellulolytic aerobes on lignocellulose digestion in the earthworm gut. Hexagons and pentagons indicate glucose (hexose) and xylose (pentose) derived from lignocellulose; yellow and brown triangles indicate microbial and earthworm enzymes, respectively. Cel, a cellulolytic aerobe; Anae, an anaerobe residing in the earthworm gut.

enzymes are resistant to proteolytic inactivation and are able to continue the saccharification reaction in animal intestine juice for several hours, which corresponds to the food transit time of earthworms [9,13]. As described above, the growth of cellulolytic as well as other aerobes is halted by anaerobiosis, causing these microorganisms to become a minor population in the gut; however, some of them are resistant to digestion and finally excreted as a part of the cast, because earthworm digestive fluid contains a microbicidal activity that is selective for certain species but neutral or stimulating for the

growth of others [6,20]. More detailed studies are needed to unveil the mechanisms by which aerobes contribute to lignocellulose digestion in the anoxic gut.

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Competing interests. None declared.

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