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, A. heteropoda (Megascoleidae) and E. 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 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, 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 Fuji 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 (NH4)2SO4/l; 0.3 g (NH2)2CO/l; 2.0 g KH2PO4/l; 0.3 g (NH4)2S/l; 0.4 g CaCl2·2H2O/l; 5.0 mg FeSO4·7H2O/l; 2.2 mg MnSO4·5H2O/l; 1.4 mg ZnSO4·7H2O/l; 3.7 mg CoCl2·6H2O/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 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 Grynowicz [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 × 10⁷ CFU (g-gut)⁻¹ and 1.5 × 10⁷ CFU (g-gut)⁻¹ for A. heteropoda and E. fetida, respectively.
After a 2-week cultivation, microbial colonies with various morphologies emerged on the filter paper, and the concentrations of microorganisms were $8.5 \times 10^3$ CFU (g-gut)$^{-1}$ and $2.1 \times 10^4$ CFU (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 1. Accession numbers for 16S rDNA (bacterial isolates) and ITS region DNA (fungal isolates) sequences**

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After a 2-week cultivation, microbial colonies with various morphologies emerged on the filter paper, and the concentrations of microorganisms were $8.5 \times 10^3$ CFU (g-gut)$^{-1}$ and $2.1 \times 10^4$ CFU (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.

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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-
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.
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

![Phylogenetic trees of cellulolytic isolates and related species constructed using the neighbor-joining method.](image)

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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 [3,8], few studies have isolated or identified their counterparts in the earthworm gut. However, metagenomic studies have begun to shed light on microbial ecology, especially regarding the bacterial biota, in the earthworm gut. 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 andoxic 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, xylene, 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
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

![Proposed hypothesis for the role of cellulolytic aerobes on lignocellulose digestion in the earthworm gut.](image-url)
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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References


