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Gut Microbiota of *Pheretima heteropoda* and *Allolobophora japonica* Fed under the Same Laboratory Conditions

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Our previous study reported that the gut microbiota of Megascolecidae was distinguishable from that of Lumbricidae. In this study, two earthworms, *Pheretima heteropoda* (Megascolecidae) and *Allolobophora japonica* (Lumbricidae), were fed under the same *in vitro* conditions to investigate the effect of feed on gut microbiota. Their gut microbiota was analyzed by denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments. Most of the dominant bands were shared between both earthworm species, but some were specific to one species. These banding patterns corresponded to *in situ* earthworms, i.e. they were directly analyzed after collection from the field. The predominant bacterium found in *P. heteropoda* gut samples was *Bacillus longiquaesitum* (AM747042)–related, whereas the predominant bacteria found in *A. japonica* gut samples were bacterium TG141 (AB308367)–related and *Rhizobium* sp. BBCT66 (DQ337551)–related. These results indicated that the major bacterial species were not influenced by the surrounding environment, including the food source, even though a large portion of the bacterial population was opportunistic.

Key words: *Allolobophora japonica*, earthworm, feeding experiment, gut microbiota, *Pheretima heteropoda*

INTRODUCTION

The beneficial effects of earthworms on soil properties are believed to be related to the close relationship that exists between earthworms and the microorganisms found in their gut and the surrounding soil (Brown, 1995). Many studies have been conducted to analyze the gut microbiota of Lumbricidae, which mainly inhabits Europe (ex. Monroy *et al.*, 2008), but few have been reported for Megascolecidae, a major family of earthworms found in Japan (ex. Khambata and Bhat, 1957; Wüst *et al.*, 2009; Nakamura and Sakai, 2011; Depkat-Jakob *et al.*, 2013). Within the soils of Japan, the majority of earthworms are of the species *Pheretima* belonging to the Megascolecidae, with only a few reported species belonging to the Lumbricidae (Aoki, 1973; Nakamura, 1968). Previously, we reported that the gut microbiota of the Megascolecidae (*Pheretima heteropoda* and *Pheretima hilgendorfi*) was distinguishable from that of the Lumbricidae (*Allolobophora japonica*), both of which were collected from the same Japanese grassland soil (Nakamura and Sakai, 2011). In addition, our results suggested that the gut microbiota might be influenced by the alimental canal structure rather than by their ecological category; however, we were unable to prove this hypothesis owing to the complex nature of the field samples. Many studies have reported that earthworms have distinct gut microbiota that is predominantly determined by their environment (ex. Furlong *et al.*, 2002). However, one study by Toyota and Kimura (2000) reported that the indigenous

microbial community in the gut of *Eisenia fetida* (Lumbricidae) was independent of habitat.

In this study, to investigate effect of feed on the gut microbiota of Megascolecidae and Lumbricidae species, the gut contents from two earthworms, *P. heteropoda* (Megascolecidae) and *A. japonica* (Lumbricidae), fed the same food under *in vitro* conditions were analyzed.

MATERIALS AND METHODS

Feeding experiment

To feed the earthworms, soil and manure were obtained from cattle grazed grassland at Kyushu University, Oita, Japan. The feeding experiment was performed according to Nakamura (1977), with a few modifications, to investigate the effect of soil bacteria. Soil was sieved (<4 mm) and stored at 4°C until further use. Manure was collected from the soil surface, air-dried, mechanically fragmented into pieces, and sieved (<4 mm). Experimental medium (food) for feeding the earthworms comprised soil and manure (1:1 (v/v)).

Adult earthworms (*Pheretima* (Goto et hatai) and *Allolobophora* (Michaelsen)) were also collected from cattle grazed grassland at Kyushu University. Species identification was conducted on the basis of characteristic morphological features (Ohfuchi and Yamaguchi, 1965; Nakamura, 1999). Earthworms were washed three times with sterile deionized water, and kept at 16.8°C in containers with the experimental medium for 3 days. As reviewed by Brown (1995), earthworms have various gut transit times of up to 20 hours. Three days (72 hours) was found to be sufficient time for food transit in this study.

The feeding experiment was conducted in Petri-dishes (9 cm in diameter), containing the experimental

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medium (15.4 g/ dish), that were incubated at 16.8°C for 3 days (one earthworm/dish). This temperature corresponded to the average soil temperature of the grassland from which the earthworms were collected. The soil moisture content was maintained at around 60% (dry wt). The feeding experiment was conducted twice under the same conditions. The earthworms used in the first experiment included 11 *P. heteropoda* and 23 *A. japonica*. The earthworms used in the second experiment included 22 *P. heteropoda* and 21 *A. japonica*.

Analysis of the gut bacterial community structure

Gut samples were prepared after 3 days of feeding by first washing all earthworms with sterile deionized water three times and then immediately freezing the worms at -20°C until further use. Earthworms were dissected and the gut (gut contents and gut wall) behind segment XX (*P. heteropoda*) or the clitellum (*A. japonica*) was equally divided into three parts (fore-gut, mid-gut and hind-gut).

Total DNA was extracted from the earthworm gut samples and experimental medium using the Fast DNA Spin Kit for soil (Qbiogene Inc., Carlsbad, CA, USA). For further purification, a GENECLEAN® Turbo Kit (Qbiogene Inc.) was used. The DNA concentration was estimated by visual comparison with authentic fragment DNA (λ /HindIII digest) after electrophoresis on an agarose gel. Purified DNA was stored at -20°C until use.

PCR and denaturing gradient gel electrophoresis (DGGE) were performed as described previously (Nakamura and Sakai, 2011). A fragment of the 16S rRNA gene (c. 194 bp) was PCR amplified from a representative, diluted, purified DNA template using the forward primer, 357f-GC, and the reverse primer, 518r (Muyzer *et al.*, 1993). The PCR reactions were performed in 25 or 50 μ l (final volume) mixtures containing 20 mM of each primer and Premix Taq [Ex Taq Version] (TaKaRa, Otsu, Japan). Reactions were incubated in a Dice thermal cycler (TaKaRa) using the following protocol: an initial denaturation step for 3 min at 94°C; then 30 cycles of 1 min at 52°C (annealing), 1 min at 72°C (elongation), and 1 min at 94°C (denaturation); followed by 1 min at 52°C and 10 min at 72°C. DNA amplification was verified using electrophoresis of the PCR products in 1.5% agarose gels.

DGGE was performed with the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. PCR products (c. 100 ng) were loaded onto 1 mm polyacrylamide gels (8% wt/vol) containing a 30%–60% linear gradient concentration of denaturant; 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide. Gel electrophoresis was conducted in 1×TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-EDTA; pH 8.0) at 200V and 60°C for 5 h. Gels were stained for 30 min with 1:10,000 (vol/vol) SYBR® Green I (TaKaRa) and photographed under ultraviolet (UV) transillumination.

RESULTS AND DISCUSSION

DGGE analysis of the gut content of earthworms fed under laboratory conditions revealed characteristic bacterial community structures for each earthworm species, with profiles distinguishable from the experimental medium (i.e. food) (Fig. 1). The profiles showed three major groups of bands. The first group was predominant in only one of the earthworm species: band 4 (*Bacillus longiquaesitum* (AM747042)-related) in *P. heteropoda* gut samples; and bands 9 and 10 (*Bacterium* TG141 (AB308367)-related), 12 (*Rhizobium* sp. BBCT66 (DQ337551)-related) and 16 in *A. japonica* gut samples. Interestingly, bands 4, 9, 10, 12 and 16, which predominated in one earthworm gut sample only, showed the same migration behavior as previous field samples (Nakamura and Sakai, 2011). The second group was shared by both earthworm species and comprised bands 1, 5 (*B. longiquaesitum* (AM747042)-related), 6, 7, 11, 13, 14 and 15. Among these, bands 1, 7 and 14 were derived from the experimental medium and their intensity was higher in gut samples. The third group, comprising bands 3 and 8, predominated in the experimental medium but diminished in intensity during passage through the earthworm intestines. Table 1 shows a comparison of the dominant bands identified in this study with those of a previous study.

DGGE profiles revealed that many dominant bands were present in both earthworm species fed on the same experimental medium under the same laboratory conditions, suggesting that establishment of the earthworm gut microbiota is dependent on the ingested material. These observations were also reported by Knapp *et al.* (2009) in *Lumbricus rubellus*. Although many bands

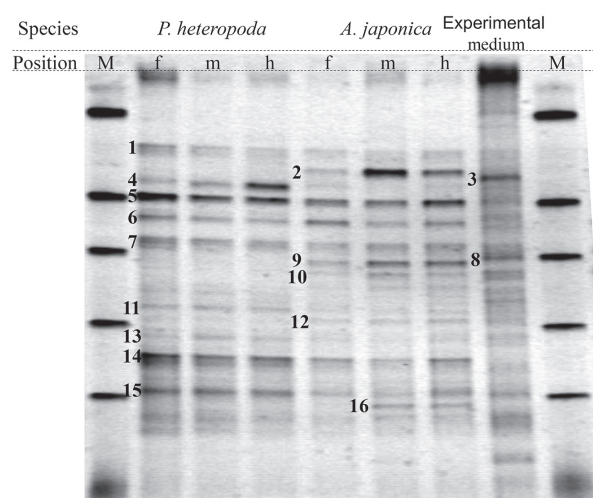


Fig. 1. Typical DGGE profiles for gut samples from *P. heteropoda* and *A. japonica*: f, fore-gut; m, mid-gut; h, hind-gut. Electrophoresis markers (M) were included. Common bands observed in most lanes (1–16) are numbered in the figure. Band 2 indicates the earthworm 18S rRNA gene sequence identified in our previous report (Nakamura and Sakai, 2011).

Table 1. Dominant DGGE bands detected in this study

Band	<i>P. heteropoda</i> gut	<i>A. japonica</i> gut	Experimental medium	Field samples*	Closest relative in DDBJ database (accession no.)	% Similarity	Phylogenetic group
1	○	○	○		–	–	–
3			○		–	–	–
4	○			○	<i>Bacillus longiquaesitum</i> (AM747042)	99.6	<i>Firmicutes</i>
5	○	○		○	<i>Bacillus longiquaesitum</i> (AM747042)	99.7	<i>Firmicutes</i>
6	○	○		○	<i>Bacillus longiquaesitum</i> (AM747040)	97.6	<i>Firmicutes</i>
7	○	○	○		–	–	–
8			○		–	–	–
9		○		○	Bacterium TG141 (AB308367)	93.4	<i>Bacteria</i>
10		○		○	Bacterium TG141 (AB308367)	93.4	<i>Bacteria</i>
11	○	○			–	–	–
12		○		○	<i>Rhizobium</i> sp. BBCT66 (DQ337551)	90.9	<i>Proteobacteria</i>
13	○	○			–	–	–
14	○	○	○		–	–	–
15	○	○			–	–	–
16		○		○	–	–	–

Note: 1)* Detected in Nakamura and Sakai (2011).

2) Band 2 indicates the earthworm 18S rRNA gene sequence identified in our previous report (Nakamura and Sakai, 2011).

were shared, some bands were specific to each species indicating that species-specific bacteria stably inhabit the earthworm gut, irrespective of their food source. The *Proteobacteria* that predominated in *A. japonica* gut samples in this study were consistent with the results of a previous study on field samples (Nakamura and Sakai, 2011), and were also found to dominate the gut contents of Lumbricidae species, such as *Lumbricus terrestris* (Schönholzer *et al.*, 2002) and *L. rubellus* (Knapp *et al.*, 2009). There may be factors that activate these bacterial species, for example, the existence of intestinal caecum or the secretion of mucus.

Collectively, this study showed that most of the dominant bacteria were shared between the two earthworm species, *P. heteropoda* and *A. japonica*. Furthermore, the dominant bacteria derived from the feed material. Our findings suggest that a large portion of the gut bacterial population is opportunistic and depends on the feed material. However, some bacteria were specific to particular earthworm species. These findings showed that the gut bacterial species were not influenced by the surrounding environment, indicating that each earthworm species possess indigenous characteristics that can influence the gut bacteria. Further elucidation of these characteristic features will aid our understanding of the interaction between bacteria and earthworms.

AUTHOR CONTRIBUTIONS

K. Nakamura designed the study, performed the experiments, analyzed the data and wrote the paper. K. Sakai supervised the work and wrote the paper. All authors assisted in editing of the manuscript and

approved the final version.

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