Isolation, characterization and practical significance of cellulose degrading bacteria from the gut wall of two ecologically distinct earthworms

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This study has determined the relationship of culturable community of cellulose degrading bacteria (CDB) within the gut walls of two habitat-specific earthworm species, epigeic (compost heap inhabitant) earthworm, *Perionyx excavatus* (PE) and an endogeic (submerged rice field inhabitant), *Glyphidrilus spelaeotes* (GS) and their functional significance. The 16Sr RNA analysis for the isolated CDB from two ecologically distinct earthworms clearly showed the presence of distinct communities of CDB in their gut ecosystem. Enzymatic assay of cellulase for the isolated CDB showed significantly higher cellulase activity compared to the reference strain M-23, *Cellulomonas cellulans* ($P < 0.01$, one-way ANOVA). The functional significance of such high cellulase activity was also demonstrated by the enhancement of decomposition of rice straw and fresh vegetation biomass in the presence of native microbiota community. The growth rate of CDB of epigeic PE was approximately twice slower than that of CDBs of endogeic GS. The CDB of PE exhibited 12 polymorphs of esterase isozyme as against 4 polymorphs for CDB of GS. The present study emphasizes the functionally significant relationship of gut wall CDB and host earthworm for sustaining efficient C-cycling in agroecosystems.

Keywords: Cellulase activity, easterase isozyme, earthworm–microorganism interactions, *Glyphidrilus spelaeotes*, intestinal microbiology, soil fauna.

EARTHWORMS are 'keystone species' in soil food webs and 'ecosystem engineers' in soils[1,2]. The earthworm gut hosts an array of microorganisms[3–9]. Earthworms and microorganisms are inter-dependent and their interactions regulate the biogeochemistry of terrestrial soils through their direct influence on soil carbon dynamics, nutrient cycling and soil physical conditions[2,10]. The evolutionary relationship between earthworm burrowing and feeding habits, and the gut wall-associated microorganisms indicated that the determinants for selection of the gut wall-associated bacterial community in common earthworm species are in the order of ecological group > habitat > species[11]. This means that the earthworm gut wall-associated microbial community may share a symbiotic or a mutualistic metabolic relationship with their host. Cellulose degrading bacteria (CDB) is an important community and carries out critical function in host nutrition by enhancing metabolite acquisition, synthesis or catabolism. Earthworms mainly consume lignocellulosic complex substances of ingested residues or soil organic matter from which they derive their energy[12]. This study is aimed at determining the occurrence of functionally significant CDB within the gut wall of earthworms and to test if such associations are dependent on the earthworm habitat.

From a practical point of view, knowledge on occurrence and screening of efficient CDB within the gut wall of earthworms may help in efficient cycling of organic residues either in field condition or in compost making process. In agro-ecosystems under tropics and subtropics, cellulose-rich rice straw (cellulose content 32% w v^{-1}) under rice–rice cropping system needs to be decomposed quickly for efficient release of nutrients. In the summer rice (March–June) to winter rice (July–December) cropping system, prevalent in the North East India, the summer rice straw needs to be recycled 20–30 days prior to transplanting of winter rice. It has been reported that the endogeic earthworm, *Glyphidrilus spelaeotes* (GS) in submerged rice field can decompose rice straw under the favourable temperature of July–August[13]; but, the cycling of winter rice straw in field condition is slowed down due to low temperature in winter months. Although there is a possibility of utilization of winter rice straw in composting using the epigeic earthworm, *Perionyx excavatus* (PE), a predominant species in aerobic composting system and/or gut wall CDB of PE, but no information is available on the gut wall microbiota of GS and their

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possible interactive role in rice straw decomposition. Earlier, it was reported that *Glyphidrilus* spp. are inhabitants of rice fields in Thailand and might play an important role in the development of rice farming[14]. The occurrence of *Glyphidrilus* spp. as endogeic earthworm in the rice fields of Kerala is also reported[15]. Therefore, the relationship between culturable community of CDB within the gut wall and earthworm ecological group and growth rate, cellulase activity, esterase isozyme as well as the ability of these CDB to accelerate decomposition of rice residue and vegetation biomass in microcosm experiment, were determined in this study.

Methods

Earthworm species and gut wall sample preparation

Two earthworm species, viz. PE (an epigeic and inhabitant of compost heap) and GS (an endogeic and inhabitant of submerged rice fields in Assam, India), were selected for this study. The individuals of GS are found in abundance in small isolated heaps of rice straw mix soils of submerged rice fields. These isolated heaps also contain worm casts (Supplementary Figure 1). Ten mature adults of each of the GS and PE were collected from submerged rice fields and compost heaps respectively (Supplementary Figure 1). The earthworms were washed with sterile distilled water to make it free from adhered soil or debris and allowed to regurgitate for 48 h by repeated changes of sterile water in the petri dish. The starved earthworms were surface-sterilized with 0.1% $HgCl_2$ for 1 min and killed by brisk immersion in sterile water at 48°C and dissected immediately under aseptic condition in a laminar flow cabinet. The exposed intestine after starvation was washed as per procedure described previously[16] with slight modification. In brief, the exposed intestines (mid plus hind gut) of ten adults of each species (~2.5 g) were pooled together and placed separately in a 15 ml capacity sterile falcon tube containing 5.0 ml sterile phosphate buffer saline (pH 7.0) and vortexed vigorously. The intestine was repeatedly washed five times. Finally, the washed intestine (hereafter termed as gut wall, 1 g) was placed in sterile falcon tube containing 10 ml of sterile phosphate buffer saline and vertexed for 30 min at 200 rpm. The serial dilution spread plate technique was used to isolate CDB using Omeliansky's agar[17]. The plates were then incubated at 30°C till the appearance of colonies. The colonies were selected on the basis of speed of their appearance and distinctness in terms of morphological features.

Intrinsic antibiotic resistance profile

The intrinsic antibiotic resistance profile (IARP) of the CDB isolate was determined using six antibiotics (tetracyclin, rifampicin, kanamycin, streptomycin, gentamycin and ampicillin) and each antibiotic at concentrations 0, 10, 20, 30, 40, 50, 100, 150, 200, 250 and 300 ppm by the paper disc method[18]. Inhibition of growth around the paper disc indicated sensitive reaction while growth around the paper disc indicated resistance reaction. This pattern of growth inhibition was recorded after 30 h of incubation.

Cellulase activity

Each CDB isolate was multiplied using Omeliansky's broth (100 ml) in conical flask (250 ml capacity) aided with buck stopper (Whatman, UK) at 30 ± 1°C. The cellulase activity was measured at five different time points (4, 8, 12, 16 and 28 days) and at each time point, three inoculated flasks and one uninoculated control flask were used for each CDB isolate. An aliquot (10 ml) of culture broth was withdrawn from flask to a sterile falcon tube (15 ml capacity) and centrifuged at 4000 rpm for 5 min, then a 0.5 ml clear solution was withdrawn and put into a test tube, which served as crude enzyme extract. Thin strips of Whatman no. 1 filter paper (32 mg) were immersed in crude enzyme extract and the mixture incubated at 50°C for 1 h. Immediately after removing the enzyme substrate mixture, 0.5 ml dinitro salicylic acid (DNS) was added and boiled in a water bath for 5 min. In the warm tubes, potassium sodium tartarate solution (10 ml) was added, cooled to room temperature and the volume reduces to 5 ml by adding sterile distilled water. The absorbance was measured at 540 nm in a spectrophotometer (Spectrascan UV-2600, Thermo Scientific, USA). A standard curve of glucose in the concentration range of 50–1000 μg ml^{-1} was also prepared for determining the crude cellulase enzyme activity as glucose equivalent. A reference strain (*Cellulomonas cellulans* M-23) from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh (India) was also taken for determination of cellulase activity.

Esterase isozyme

Soluble protein was extracted from CDB isolates and electrophoresed in 12% (w v^{-1}) separating the gel as previously described[19]. Isozyme of the enzyme esterase was analysed for all CDB isolates using native PAGE. Microbial isolates were grown in 50 ml of nutrient broth to obtain optical density (OD) of 0.6 at 600 nm wavelength in an environmental shaker at 180 rpm and 32°C. The cells were harvested by centrifugation at 10,000 *g* for 5 min at 4°C, resuspended in 5 ml of extracting buffer solution and sonicated (Labsonic® U) with microtip for 30 sec. After sonication, cell debris was precipitated by centrifugation at 10,000 rpm for 20 min at 4°C.

Growth curve

CDB isolates were grown as pure culture using Omeliansky's broth (100 ml) in an environmental shaker (30 ± 1°C and 180 rpm). After attaining log phase (~5.0×10^5 cells ml^{-1} broth) each pure culture broth was used as seeding material. To determine growth curve of an isolate, a total of 63 conical flasks (100 ml capacity) each containing 25 ml of Omeliansky's broth with 2.5 g thin strips of Whatman No. 1 filter paper instead of cellulose powder were arranged. Out of 63, 54 conical flasks were inoculated with seed culture broth (0.1 ml per flask) and the other nine flasks were kept as uninoculated control. The bacterial growth was determined at nine different time points and in each time point six inoculated flasks along with one uninoculated control tube were used. Replacing cellulose powder with filter paper eliminated the difficulty of measuring growth spectrophotometrically as cellulose powder also absorb incident light. At each time point, of the six inoculated flasks, three flasks were vortexed vigorously and the top portion of the broth poured into cuvette leaving the filter paper strip and absorbance reading taken in spectrophotometer at 600 nm. In the other three flasks, carboxy methyl cellulose (CMC, 0.1%) was added and incubated for 1 h at 30°C. After incubation, the flasks with CMC were vortexed vigorously and absorbance reading of the suspension broth and the CMC added control were determined at 600 nm in a spectrophotometer. The purpose of adding CMC was to detach microbial cells from the filter paper.

Genomic DNA extraction and polymerase chain reaction amplification

The pure culture of CDB isolate in 5 ml luria broth (optical density at 660 nm was 0.6) was centrifuged at 10,000 rpm for 5 min. The cell pellet was washed twice with sterile distilled water by re-suspending followed by centrifugation. The washed pellet was re-suspended with 90 µl sterile PCR grade water plus 10 µl lysozyme (1 mg ml^{-1}, HiMedia, India) by vortexing and incubated at 37°C for 30 min followed by 95°C for 5 min and finally centrifuged at 10,000 rpm for 2 min. The genomic DNA in the supernatant was used for PCR amplification. The absorption spectrum of DNA extracts (230–280 nm) was determined using a Nano-drop® 2000 spectrophotometer (Thermo Scientific, USA) according to the manufacturer's instructions. DNA was quantified based on absorption at 260 nm and the quality judged based on $A_{260/230}$ and $A_{260/280}$ ratios. Genomic DNA of each isolate was amplified in a Gradient Master Cycler 5331 (Eppendorf Make, Germany) with primer pair (27f and 1492r) specific to bacterial domain[20]. This primer pair is specific to 16S rRNA genes of the bacterial domain and yields amplified product size of ~1465 bp. The reactions were amplified in a 25 µl volume containing 25 ng genomic DNA as template, 100 nM of each oligonucleotide primers [27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′)], 12.5 µl of Go-*Taq* DNA polymerase (Promega,USA) along with bovine serum albumin (BSA concentration 0.1 µg $µl^{-1}$; New England Biolabs, UK) and the required quantity of PCR grade water (HiMedia, India). The PCR reaction condition included an initial 3 min denaturation at 94°C, and was followed by 30 thermal cycles of 1 min at 94°C, 1 min at 62°C and 2 min at 72°C. Amplification was completed with the final extension step at 72°C for 7 min. All PCR products were examined by electrophoresis in an agarose gel (1% w v^{-1}) using a 100 bp DNA ladder (New England Biolabs, UK).

Amplified ribosomal DNA restriction analysis

Amplified product of 16S rDNA of each isolate was restriction-digested in a 10 µl reaction volume containing 0.4 µl of *Hae*III enzyme, 1 µl of restriction enzyme buffer, 0.2 µl of BSA, 4 µl of PCR product and 4 µl of PCR grade water. Digestion was performed overnight at a constant temperature of 37°C. The digested product (10 µl) of each isolate was mixed with 2 µl of loading dye and the restriction pattern within the mixture was determined by electrophoresis in an agarose gel (2.0% w v^{-1} containing gel-red fluorochrom stain with a working strength of 0.06X, Biotium, USA) using a mini gel electrophoresis assembly (HU10, Sci-plas, Hongkong). The gel was visualized and the image captured using gel documentation system (BioRad, CA, USA).

Sequencing of 16S rRNA gene

Amplified product of the 16S rDNA of each isolate obtained using primer pair 27f and 1492r was used as the template DNA for amplification of 16S rDNA fragments at *E. coli* positions 968–1401 (V6-V8 regions of 16S rRNA gene[21], using another set of primer pair 984f (5′-AACGCGAAGAACCTTAC-3′) and 1378r (5′-CGGTGTGTACAAGGCCCGGGAACG-3′). Amplification was carried out in a 50 µl reaction volume using a Gradient Master cycler 5331 (Eppendorf Make, Germany). The reaction composition consisted of 50 ng amplified PCR product (amplified with 27f and 1492r) as template DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs (all reagents from New England Biolabs, UK), 100 nM of each of 984f and 1378r primers, 5 µl BSA (concentration 0.1 µg $µl^{-1}$), and 1U of *Taq*-DNA polymerase. PCR reaction conditions consisted of an initial denaturation step (94°C, 3 min) followed by 30 cycles of 1 min of denaturation at 94°C, 1 min at 60°C for primer annealing, and 2 min at 72°C for primer extension, followed by a final step at 72°C for 7 min, stored at 4°C. Products (length

433 bp) were first analysed by electrophoresis in an agarose gel (1% w v^{-1}). PCR products were purified using QIAquick PCR purification kit as per manufacturer's instruction (Qiagen, GmbH, Germany). Purified PCR products were ligated into pDrive Cloning Vector (Qiagen, GmbH, Germany), and transformed into QIAGEN EZ competent cells (Qiagen, GmbH, Germany). The transformants were selectively grown on LB, according to the manufacturer's instructions. White positive colonies were grown in LB plus ampicillin[22] and the plasmid DNA extracted using QIAquick Plasmid Mini Extraction Kit (Qiagen GmbH, Germany). Plasmid inserts were bi-directionally sequenced using the universal primers T7 and SP6 (Xcelris Labs Limited, Ahmedabad, India). Inserts (from 2 colonies per insert) were sequenced.

Decomposition of rice straw and fresh vegetation biomass

Four CDB isolates of each of the PE and GS and one reference strain *Cellulomonas cellulans* (M-23) used in decomposition study were selected based on their higher cellulase activity and faster growth rate. Effect of these isolates on decomposition of organic matter was determined indirectly by measuring the CO_2-C evolution in two different sets of microcosm experiments (one set for rice straw and the other for vegetation biomass as sources of organic matter). To conduct microcosm experiment for rice straw decomposition, a sandy loam soil was collected (~5.0 kg) and mixed thoroughly. The soil was passed through 1 mm sieve. Each microcosm unit was represented by a 500 ml capacity conical flask containing 1 ml broth culture (5×10^8 cells per ml) of isolate, 20 g unsterilized soil, 0.2 g finely ground rice straw (size < 100 μm) and the moisture holding capacity of the mixture is adjusted to 80%. The microcosm unit with rice straw plus soil and without broth culture served as control. CO_2-C evolution was measured at six different time points (3, 6, 9, 12, 16 and 20 h). There were three replicate microcosm units per treatment at each point of time. Hence, each set of experiment contained 180 microcosm units [10 treatments (8 isolates + 1 reference strain + 1 control) × 6 time points × 3 replicate units per time point]. The room temperature ranges of day and night of the experimental period of July were 30–34°C and 26–29°C respectively. An excess of standard alkali (10 ml of 0.2N NaOH solution) was taken in 1.8 mm (i.d.) test tube and placed inside a flask and the mouth of the flasks was made air tight with rubber cork and grease to ensure that CO_2 evolved during decomposition of organic matter is absorbed in NaOH. The destructive sampling (3 microcosm units per treatment at one time point) was done for quantification of unused part of the NaOH by titration using standard 0.1 N H_2SO_4 in the presence of excess of 5% $BaCl_2$ and phenolphthalein indicator. The experiments were conducted for 20 days. The only difference in the microcosm experiment for decomposition of fresh vegetation biomass was that each microcosm unit contained 10 g biomass (chopped into ~1 cm length) plus 1 ml of CDB pure culture (5.0×10^8 cells per ml).

Statistical analysis

All univariate statistical analyses were performed using SPSS v12.0 (statistical packages for Social Science Inc., Chicago, IL, USA). Within each parameter (cellulase activity, cumulative CO_2-C evolved at each time point, etc.) data among the treatments were normally distributed as determined using the Kolmogorov–Smirnov test and wherever normality was not observed the data was subjected to mild transformation (square root transformation). Any significant differences between treatments within a parameter were determined by ANOVA (incorporating the Levene's Statistic to test for the equality of group variances, and the Tukey's Honestly Significance Difference (HSD) test at $P < 0.05$ for pairwise comparisons).

A binary (presence–absence) matrix was created in Excel worksheet 4.0 (Microsoft Office Excel, 2003) by scoring the ARDRA fingerprint. The binary matrix thus obtained was exported to PRIMER v6.1.9 software (Primer-E Ltd, Plymouth, UK) and the dataset standardized by the total method[23]. A Bray–Curtis resemblance matrix was generated using the square-root transformed standardized binary matrix[24,25]. Dendrogram was constructed by hierarchical cluster analysis (group-average linking) using the Bray-Curtis resemblance matrix incorporating SIMPROF analysis to check significant difference within the clusters[24].

Results

Occurrence and screening of CDB within gut wall

Eight CDB isolates were selected from the culture plate (containing Omeliansky's agar supplemented with cellulose as sole carbon source) seeded with the gut wall homogenate of each of the two earthworms (PE and GS). The selection was done based on time taken for appearance of colony and colony morphology. The CDB isolates within the gut wall of PE were PE-1, PE-2, PE-3, PE-4, PE-5, PE-6, PE-7 and PE-8 and for GS were GS-1, GS-2, GS-3, GS-4, GS-5, GS-6, GS-7 and GS-8. The isolates of PE developed into full-grown colonies within 32–48 h, whereas isolates of GS took 28–36 h on Omelianksy's agar. Isolates of both the earthworm species developed into full-grown colonies within 15–24 h on nutrient agar. The IARP of the isolates obtained using 6 antibiotics as MIC is presented (Supplementary

Table 1) and the results indicated that IARP of each isolate was unique.

The reference strain M-23 (*Cellulomonas celulans*) and all CDB isolates of PE and GS showed cellulase activity in the range of 0.06–0.59 μM glucose ml^{-1} min^{-1} (Figure 1). The cellulase activities (ranged from 0.42 to 0.59 μM glucose ml^{-1} min^{-1}) of PE-2, PE-4, PE-5, PE-7, GS-1, GS-2, GS-3 and GS-7 were significantly higher than that (0.36 μM glucose ml^{-1} min^{-1}) produced by the reference strain M-23 ($P < 0.01$, one-way ANOVA). The cellulase activities of the other four gut wall isolates of each of PE and GS were comparable to that of the reference strain M-23 ($P \geq 0.05$, one-way ANOVA). Out of the 16 CDB isolates, PE-5, PE-7 and GS-3 showed the maximum cellulase activity, i.e. 0.59, 0.58 and 0.55 μM glucose ml^{-1} min^{-1} respectively. The two peaks were observed in the cellulase activity time trend for all the CDB isolates during 28 days of incubation (Figure 1). Interestingly, the two peaks of cellulase activity of CDB isolates of PE were recorded on the 8th and 16th days of incubation, whereas the two peaks were recorded on the 4th and 12th days of incubation for the CDB isolates of GS.

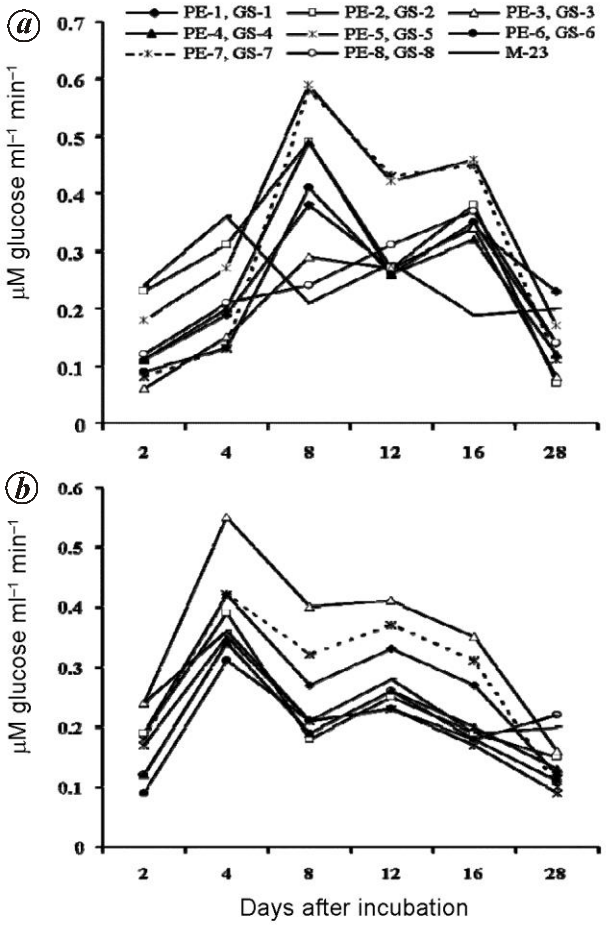

Figure 1. Cellulase activity of the gut wall-associated bacterial isolates of an epigeic earthworm species *Perionyx excavatus* (***a***), an endogeic earthworm species *Glyphidrilus spelaeotes* (***b***). Bacterial isolates were grown as pure culture in Omeliansky's broth supplemented with cellulose as sole C-source in an environmental shaker (30°C and 180 rpm) and the cellulase activitiy was determined at four days interval for a period up to 28 days in the replicated destructive broth samples and expressed as glucose equivalent. A cellulose degrading bacterium (*Cellulomonas cellulans, strain* M-23, Microbial Type Culture Collection, Chandigarh, India) was also tested for determination of cellulase activity as reference strain.

All the CDB isolates were esterase isozyme-positive (Figure 2). The esterase banding pattern of CDB isolates of PE was different from that of CDB isolates of GS. The degree of polymorphism in esterase isozyme of CDB isolates of GS was considerably less (only 4 polymorphic isozymes) compared to that (12 polymorphic isozymes) of CDB isolates of PE. The hierarchical cluster analysis performed using binary matrix (presence–absence) based on esterase isozyme banding pattern produced two distinct clusters and each cluster represented by the CDB isolates of each earthworm group (data not shown).

The CDB isolates exhibited characteristic growth pattern – a lag phase of about 50 h followed by a gradual increase in cell density and attainment of log phase at around 74 h after inoculation (Figure 3). The CDB isolates of PE (i.e., PE-2, PE-4, PE-5 and PE-7) exhibited approximately growth rate twice slower than that of GS-1, GS-2, GS-3 and GS-7 of GS. Incubation of replicate following CMC addition after each absorbance reading had no significant effect on growth rate measurement in case of PE isolates except PE-4. Whereas the absorbance values were more with CMC addition at 74 h onwards in case of the CDB isolates of GS and this effect was very prominent in case of GS-3 (Figure 3).

CDB community of gut wall-ecological group specific

The 16S rRNA genes of the 16 CDB isolates were sequenced and based on the origin of their homologues, we tentatively identified these culturable CDB isolates (BLASTn analysis, Table 1). Out of 16 CDB isolates, the occurrence of 9 CDB isolates on the gut wall of earthworms was ecological group dependent. The isolates GS-1, GS-2, GS-3, GS-5 and GS-6 were unique to the gut wall of endogeic earthworm GS and the isolates PE-1, PE-2, PE-5, PE-6 and PE-8 were unique to the gut wall of epigeic earthworm PE. On the other hand, the isolates PE-3, PE-4, PE-7, GS-4, GS-7 and GS-8 were common to both epigeic and endogeic earthworms (Table 1). The hierarchical cluster analysis performed on the binary matrix (presence–absence) based on ARDRA profiles of the 16 CDB isolates (Figure 4), clearly indicated that isolates whose occurrence was ecologically group-dependent formed two distinct clusters and the similarity between these two clusters was less than 30%. The clusters formed by the isolates common to both earthworms showed

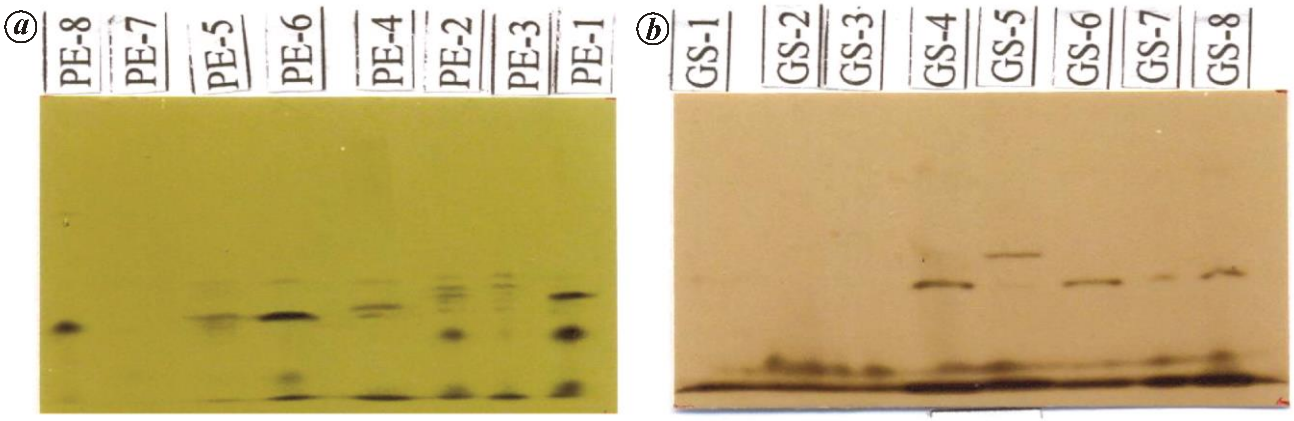

Figure 2. Comparative banding pattern of esterase isozyme exhibited by the cellulose degrading bacteria inhabitant of the gut walls of an (***a***) epigeic earthworm *Perionyx excavatus* and (***b***) an endogeic earthworm *Glyphidrilus spelaeotes*.

Table 1. Phylogeny of gut bacteria of earthworms *Perionyx excavatus* (PE) and *Glyphidrilus spelaeotes* (GS) isolated using the Omeliansky's agar supplemented with cellulose as sole carbon source, based on their 16S ribosomal DNA sequence homology

Bacterium strain ID	Ribotype[a] size (bp)	Percentage of homology with respect to 16S rRNA gene with nearest member (accession number)	Putative genus of bacterium
PE1	420	100%, *Mycobacterium monacense* strain M11 (GU142931.1)	*Mycobacterium* sp.
PE2	421	99%, *Stenotrophomonas maltophilia* strain KNUC605 (HM047520.1)	*Stenotrophomonas* sp.
PE3	419	99%, *Acinetobacter* sp. YF14 (HQ684847.1)	*Acinetobacter* sp.
PE4	417	99%, *Alcaligenes faecalis* strain DZ2 (HQ202537.1)	*Alcaligenes* sp.
PE5	423	99%, *Stenotrophomonas maltophilia* strain KNUC605 (HM047520.1)	*Stenotrophomonas* sp.
PE6	420	98%, *Chryseobacterium* sp. bk_48 (HQ538681.1)	*Chryseobacterium* sp.
PE7	420	100%, *Acinetobacter* sp. YF14 (HQ684847.1)	*Acinetobacter* sp.
PE8	415	99%, *Chryseobacterium indologenes* strain B7 (HQ259684.1)	*Chryseobacterium* sp.
GS1	426	99%, *Chromobacterium piscinae* type strain LMG 3947T (AJ871127.2)	*Chromobacterium* sp.
GS2	419	100%, *Pseudomonas aeruginosa* strain NL01 (JF331665.1)	*Pseudomonas* sp.
GS3	421	99%, *Bacillus megaterium* strain L4 (GQ479946.1)	*Bacillus* sp.
GS4	413	99%, *Alcaligenes faecalis* strain DZ2 (HQ202537.1)	*Alcaligenes* sp.
GS5	420	99%, *Sphingomonas* sp. H-14 (AB618494.1)	*Sphingomonas* sp.
GS6	419	100%, *Pseudomonas aeruginosa* strain NL01 (JF331665.1)	*Pseudomonas* sp.
GS7	420	100%, *Acinetobacter* sp. YF14 (HQ684847.1)	*Acinetobacter* sp.
GS8	418	100%, *Acinetobacter* sp. YF14 (HQ684847.1)	*Acinetobacter* sp.

[a]Ribotypes were obtained by amplification of genomic DNA 6f pure bacter5a3 culture using the denaturing gradient gel electrophoresis primer pair 984F and 1378r specific to 16S rRNA genes.
Nearest homologs were determined by BLASTn analysis against the National Centre for Biotechnology Information (NCBI) nucleotide database (www.ncbi.nlm.nih.gov).

higher similarity (at 50% or more) with the cluster formed by isolates unique to epigeic and lesser similarity (at 30%) with the cluster formed by isolates unique to endogeic (Figure 4).

CDBs of gut wall origin enhance organic matter decomposition

Inoculation of rice straw and fresh vegetation biomass with the CDB isolates from both the earthworms enhanced the production of cumulative CO_2-C significantly compared to that produced in uninoculated control during 20 days of decomposition ($P < 0.05$, one-way ANOVA; Tables 2 and 3). Inoculation of rice straw or fresh vegetation biomass with the CDB isolates PE-5 and GS-3 produced significantly higher ($P < 0.05$, one-way ANOVA) quantity of cumulative CO_2-C compared to that produced by the reference strain M-23 during 20 days of decomposition. The effects of inoculation of rice straw or fresh vegetation biomass with CDB isolates other than PE-5 and GS-3 on the production of cumulative CO_2-C were comparable or slightly higher than that produced by the reference strain M-23 but the effects were not significant ($P \geq 0.05$, one-way ANOVA).

Discussion

The two test earthworms (*Perionyx excavatus*, an epigeic and *Glyphidrilus spaleotes*, an endogeic in sub-tropical

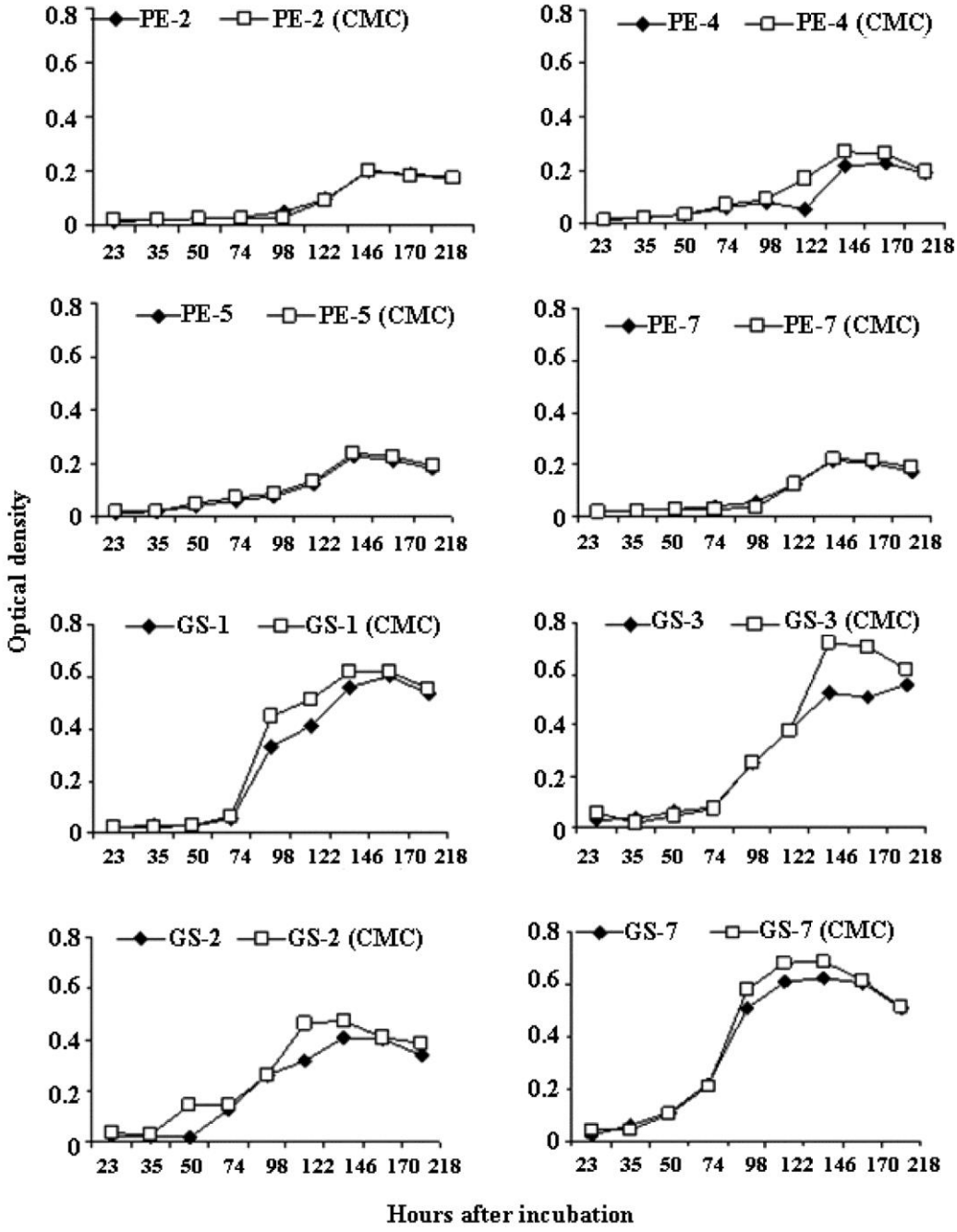

Figure 3. Growth curves of gut wall-associated bacterial isolates of epigeic earthworm *Peryonix excavatus* (PE) and endogeic *Gryphidrilus spaleotes* (GS) determined by growing in Omeliansky's broth supplemented with thin strips of Whatman No. 1 filter paper and absorbance read as 600 nm. CMC denotes carboxyl methyl cellulose.

and tropical climates) are very distinct in terms of feeding habit and the type and quality of feed resources they ingest. PE is a dweller of compost heap, and can grow and reproduce at 75.0–85.0% moisture content in composting material; whereas GS is a dweller of submerged rice field soils and can withstand saturation moisture level to submergence in soil continuously up to 15–20 days[26]. This study compared for the first time the gut wall CDB community between an epigeic earthworm of aerobic compost heap and an endogeic earthworm of submerged rice fields. The present findings provide clear evidence that the gut wall of earthworms is inhabitated by a diverse community of CDB. The interaction between CDB and earthworms is functionally significant because the cellulase activity of these CDBs was significantly higher (ranged from 0.42 to 0.59 μM glucose ml^{-1} min^{-1}) compared to that (0.36 μM glucose ml^{-1} min^{-1}) of the reference strain M-23 (*Cellulomonas cellulans*, a CDB of soil

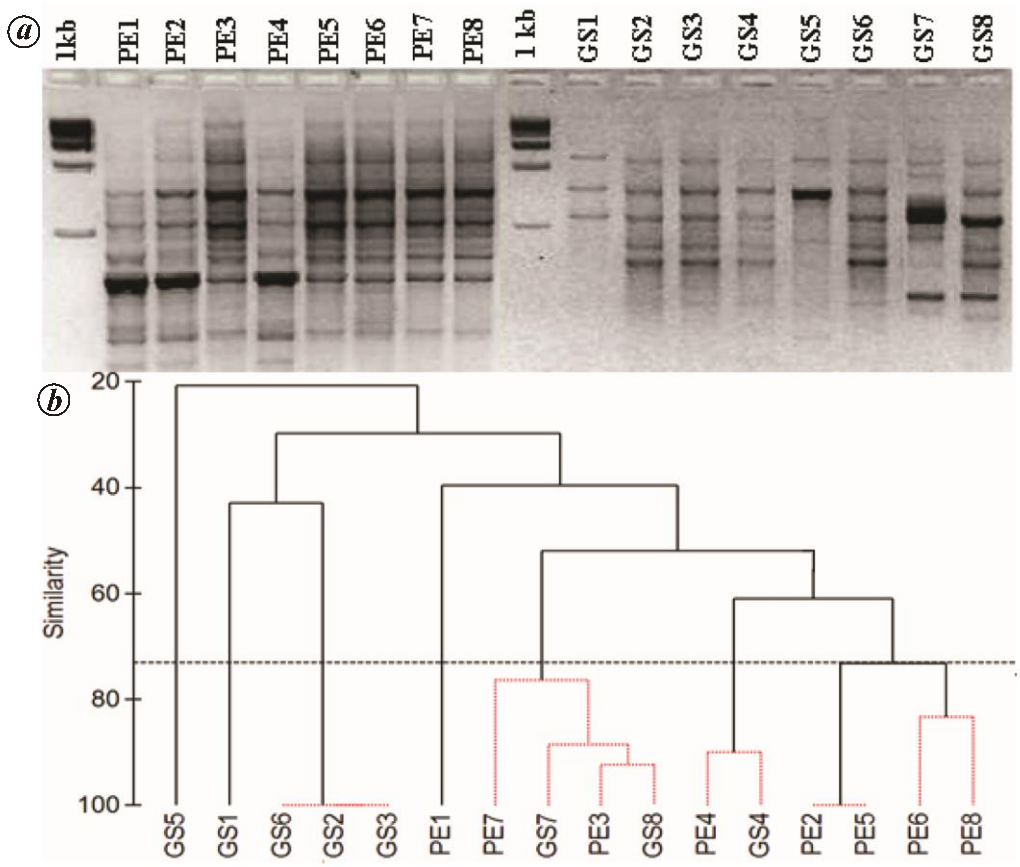

Figure 4. *Hae*III restriction pattern of PCR-amplified product of 16S rRNA gene (ARDRA) of the pure culture isolates of cellulase degrading bacteria obtained from the gut wall of the epigeic earthworm species *Perionyx excavatus* (***a***), and the endogeic earthworm species *Glyphidrilus spelaeotes*. Amplification of 16S rRNA gene of pure culture isolate was carried out using the primer pair 27f and 1492r. An equal quantity of amplified product (1 μg μl^{-1}) for each isolate was digested with *Hae*III restriction enzyme and loaded per well in the agarose gel (2.0% w v^{-1}). Hierarchical clustering of ARDRA fingerprint was performed using the Bray-Curtis resemblance matrix generated for the standardized binary matrix of ARDRA bands (with group-average linking) (***b***). Hierarchical analysis was conducted using PRIMER v6.1.9 software (Primer-E Ltd., Plymouth, UK).

Table 2. Effect of inoculation of cellulose degrading gut wall bacteria of the epigeic earthworm *Perionyx excavatus* and the endogeic earthworm *Glyphidrilus spelaeotes* on cumulative CO_2-C evolution during 20 days of decomposition of rice straw[β]

	Cumulative CO_2-C evolved (mg) Days after inoculation					
Isolates	3	6	9	12	16	20
Control	2.33[a]	4.18[a]	5.92[a]	7.12[a]	8.22[a]	9.59[a]
PE-2	2.27[a]	4.60[b]	6.98[b]	9.27[b]	10.19[b]	10.90[b]
PE-4	2.31[a]	4.92[b]	7.31[b]	9.67[b]	10.67[b]	11.50[bc]
PE-5	2.29[a]	4.80[b]	7.34[b]	9.75[b]	10.73[b]	11.69[c]
PE-7	2.36[a]	4.82[b]	7.30[b]	9.63[b]	10.72[b]	11.47[bc]
GS-1	2.37[a]	4.70[b]	7.01[b]	9.25[b]	10.34[b]	10.67[b]
GS-2	2.27[a]	4.75[b]	7.11[b]	9.42[b]	10.23[b]	10.94[b]
GS-3	2.25[a]	4.82[b]	7.31[b]	9.77[b]	10.83[b]	11.64[c]
GS-7	2.22[a]	4.71[b]	6.99[b]	9.22[b]	10.51[b]	10.72[b]
M-23	2.18[a]	4.73[b]	6.98[b]	9.36[b]	10.40[b]	10.61[b]

[β]Each microcosm unit represented by a 500 ml capacity conical flask containing 1 ml broth culture (5.0×10^8 cells ml^{-1}) of isolate plus 20 g unsterilized soil plus 0.2 g finely ground rice straw (size <100 μm) and the exception in the control microcosm unit was without inoculant.

Values within a column that differ significantly ($P < 0.01$, based Tukey's HSD test within one-way analysis of variances) are followed by different letters.

Table 3. Effect of inoculation of cellulose degrading gut wall bacteria of the epigeic earthworm *Perionyx excavatus* and the endogeic earthworm *Glyphidrilus spelaeotes* on cumulative CO_2-C evolution during 20 days of decomposition of fresh vegetation biomass[β]

	Cumulative CO_2-C evolution (mg) Days after inoculation					
Isolates	3	6	9	12	16	20
Control	2.38[a]	4.95[a]	8.68[a]	11.90[a]	14.72[a]	17.81[a]
PE-2	2.68[a]	6.17[e]	9.60[bc]	13.16[bc]	16.16[b]	20.65[bc]
PE-4	2.33[a]	5.55[bc]	9.28[b]	13.02[bc]	15.86[b]	19.93[bc]
PE-5	2.65[a]	5.87[de]	9.85[c]	13.48[bc]	16.38[b]	20.98[c]
PE-7	2.45[a]	5.47[bc]	9.25[b]	12.98[bc]	16.02[b]	20.23[bc]
GS-1	2.28[a]	5.33[b]	9.11[b]	12.90[b]	15.72[b]	19.61[b]
GS-2	2.43[a]	5.88[de]	9.99[c]	13.63[c]	16.46[b]	19.78[b]
GS-3	2.96[a]	7.16[f]	11.22[d]	14.84[d]	17.57[c]	20.98[c]
GS-7	2.38[a]	5.71[cd]	9.44[b]	13.07[bc]	16.06[b]	20.49[bc]
M-23	2.62[a]	6.10[e]	9.64[bc]	13.22[bc]	16.26[b]	20.84[c]

[β]Each microcosm unit contained 10 g biomass (chopped into ~1 cm length) plus 1 ml of broth culture (5.0×10^8 cells ml^{-1}) and the exception in the control microcosm unit was without inoculant.
Values within a column that differ significantly ($P < 0.01$, based Tukey's HSD test within one-way analysis of variances) are followed by different letters.

origin). Moreover, the CBD isolates within the gut walls were positive for esterase isozyme. Among the whole array of enzyme markers, the esterase was well suited for this investigation as micro-organisms that hydrolyse the ester linkage between phenolic acids and polysaccharides of plant cell walls are potential sources of enzymes for degradation of lignocellulosic residues[26,27]. Interestingly, the zymogram of esterase isozyme indicated 12 and 4 isozyme bands in the gut wall CDB of PE and GS respectively. The litter materials are composed of highly diverse forms of lingocellulosic substances of various plant origins; whereas soil organic matter consists of unrecognizable partially decayed plant residues due to the action of litter transformers and agents of micro-food-web in soil that lead to the production of humic substances[12]. In order to digest or degrade more complex and diverse lignocellulosic substances in ingested litter material, the metabolic needs of the epigeic earthworm PE support the development of gut wall resident CDB community characterized by higher degree of polymorphism in esterase enzyme complex. In contrast, the endogeic earthworm GS ingests primarily soil organic matter along with soil particles of rice fields characterized by less diverse and relatively uniform lignocellulosic substances and hence, the metabolic needs of the endogeic earthworm support the development of gut wall resident CDB community characterized by low degree of polymorphism in esterase isozyme complex. This preliminary result needs further confirmation through well-designed future research plan.

We were interested to screen the gut wall of the CDB having faster growth and higher level of cellulase activity and their ability to carry out mineralization of cellulose so that in future we could examine if the inoculation of efficient CDB along with earthworm species can speed up decomposition of rice residues in field conditions. The growth pattern of each group of CDB indicated not only their distinctness in terms of their identity but also varies in mechanism by which they utilize cellulose. In general, the gut wall CDBs of PE was slow growing than those of GS. Addition of CMC to the broth culture caused increase in absorbance reading to varying extent depending on the type of the CDB isolate. The cell density of the gut wall of CDB of PE was not affected by CMC except PE-4, whereas that of the gut wall of CDB of GS increased during 74 to 146 h of incubation. The reason why CMC did not cause a change in cell density of CDB of PE might be due to the fact that these isolates did not adhere strongly on cellulose strips. This could also explain the varied efficiency of cellulose utilization as exhibited by the relatively lower cellulase activities exhibited by gut isolates from PE compared to those of GS. The gut wall CDB of GS adhered to cellulose strips with higher tenacity and thus were efficient degrader of cellulose. This can also be supported by their better growth rate and cellulase activity. It was earlier reported that utilization of cellulose would require efficient adsorption of microbial cells upon cellulose substrates and CMC is able to detach microbial cells from the cellulose substrate but does not affect their growth[28].

The complete hydrolysis of insoluble cellulose requires the synergistic action of endo-β-glucanase, exo-β-glucanase and β-glucosidase components of cellulase enzyme complex[12]. Cellulase varies widely in proportions of these components. Enzyme preparation lacking exo-β-glucanase would scarcely hydrolyse insoluble cellulose but would readily breakdown soluble derivatives such as CMC. Filter paper is insoluble as it is neither too susceptible nor too resistant to cellulase[29]. Soluble CMC is mainly suitable for the assay of endoglucanase activity,

whereas insoluble substrates, like cotton or filter paper are suitable for determination of combined activity of endo- and exo-glucanases. The activity of cellulase system of the gut wall CDB isolates of both the earthworms produced appreciable amounts of reducing sugars, estimated as glucose equivalents. It ranged from 0.06 to 0.59 μM glucose ml^{-1} min^{-1}, which is comparable to the values reported by the past study on cellulase assay[30]. The two peaks in the time trend of the activity of cellulase complex of the CDB isolates in broth culture can be attributed to the fact that there exists complex enzymatic mechanisms by which product inhibition occur. In enzymatic assays carried out with industrial strains and various mutants with high cellulase activity, such phenomenon was found to be masked[31]. It is generally expected that cellobiose is an intermediate product of non-reducing ends of the cellulose chain. In the final step, *β*-glucosidase hydrolyses glucose chain fragments to glucose and the products can inhibit the activity of the cellulase system. The drop of cellulase activity between peak 1 and peak 2 and then gradual drop after peak 2 (Figure 1) can be attributed to product inhibition by cellobiose and glucose respectively. As the cultures were grown in a closed system (500 ml flask) the CDB isolates of PE showed a burst in cellulase activity up to the 8th day and the subsequent increase in concentration of cellobiose from the 8th day inhibited cellulase activity. By 28th day, the isolates were perhaps in the death phase and could not produce cellulase any more. In contrast, the CDB isolates of GS showed the highest cellulase activity on the 4th day and the product inhibition due to cellobiose was observed on the 8th day. CDB isolates of GS attacked the cellulose substrate faster and thus they are likely to be quicker decomposers of cellulose compared to CDB isolates of PE. Results of the microcosm experiments indicated that the gut wall CDB isolates could decompose rice straw or fresh vegetation biomass in the presence of native microbiota. This was evident from the values of cumulative CO_2-C evolution during 28 days of decomposition. Therefore, it can be stated that the gut wall of earthworms harbours functionally significant CDB community. Moreover, there is scope for efficient recycling of crop residues in field condition as well as in composting process through co-application of the gut wall CDB as potential bioinoculants and their host earthworm.

Analysis of 16S rRNA gene fragments of the culturable bacteria within gut walls indicated that the occurrence of some members of the CDB community clearly separates into two distinct ecological groups. For example, the isolates in the genus *Mycobacterium*, *Stenotrophomonas* and *Chryseobacterium* were unique to epigeic PE and the isolates in the genus *Chromobacterium*, *Pseudomonas*, *Bacillus* and *Sphingomonas* were specific to endogeic GS. The isolates in the genus *Acinetobacter* and *Alcaligenes* were common in both the earthworms. Though many past studies confirmed the occurrence of members of the above genus in the earthworm gut environment, i.e. gut content[3,8,32,33], we found that it was difficult to interpret whether the occurrence of these bacteria was casual association from the ingested feed resources/soil or the resident bacteria with functional significance. In the present study, isolates were obtained from the starved and repeatedly washed intestines and hence, can be considered as tightly associated members of the gut wall community[7,11]. Therefore, our study corroborates the findings of a recent study conducted on evolutionary relationship of gut wall bacterial community and earthworm ecological group in temperate climate that common earthworm ecological groups foster the development of distinct gut wall-associated bacterial communities[11]. Such distinctness of the CDB community within the gut wall according to ecological group might be the reflection of the habitat characteristics, type and quality of feed resources ingested[8,9].

In conclusion, this study confirmed that earthworms support the resident CDB community within their gut wall. Moreover, majority of the characterized CDBs exclusively formed two distinct ecological groups based on the environments the two earthworms inhabited. This relationship is also functionally significant as the members of the gut wall CDB community exhibited different levels of cellulase activity and easterae isozyme polymorphism depending on the kind of food particles the earthworms foraged on from their respective habitat. More screening of efficient members of the gut wall CDB community of earthworms of diverse habitats is likely to develop potential bioinoculants for quick recycling of crop residues in field conditions as well as in composting process. Advancement on the understanding of the relationship between gut wall CDB community and earthworm ecological group through this research will maximize the ability to explore the benefits of earthworm–microorganism interactions for sustaining efficient C-cycling in agroecosystems.

1. Jones, C. G., Lawton, J. H. and Shachak, M., *Organisms as Ecosystem Engineers, in Ecosystem Management*, Springer, 1996, pp. 130–147.
2. Brown, G. G., Barois, I. and Lavelle, P., Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. *Eur. J. Soil Biol.*, 2000, **36**(3), 177–198.
3. Furlong, M. A. *et al.*, Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Appl. Environ. Microbiol.*, 2002, **68**(3), 1265–1279.
4. Schönholzer, F., Hahn, D. and Zeyer, J., Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. *FEMS Microbiol. Ecol.*, 1999, **28**(3), 235–248.
5. Ihssen, J. *et al.*, N_2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. *Appl. Environ. Microbiol.*, 2003, **69**(3), 1655–1661.
6. Schramm, A. *et al.*, Acidovorax-like symbionts in the nephridia of earthworms. *Environ. Microbiol.*, 2003, **5**(9), 804–809.

7. Singleton, D. R. *et al.*, Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). *Soil Biol. Biochem.*, 2003, **35**(12), 1547–1555.
8. Egert, M. *et al.*, Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L.(Oligochaeta: Lumbricidae). *FEMS Microbiol. Ecol.*, 2004, **48**(2), 187–197.
9. Knapp, B. *et al.*, Application of denaturing gradient gel electrophoresis for analysing the gut microflora of *Lumbricus rubellus* Hoffmeister under different feeding conditions. *Bull. Entomol. Res.*, 2008, **98**(03), 271–279.
10. Lattaud, C. *et al.*, Activities of the digestive enzymes in the gut and in tissue culture of a tropical geophagous earthworm, *Polypheretima elongata* (Megascolecidae). *Soil Biol. Biochem.*, 1997, **29**(3), 335–339.
11. Thakuria, D. *et al*., Gut wall bacteria in earthworms: a natural selection process. *ISME J.*, 2010, **4**, 357–366.
12. Howath, W., Carbon cycling and formation of soil organic matter. In *Soil Microbiology, Ecology and Biochemistry* (ed. Paul, E. A.), Academic Press, Canada, 2007, 3rd edn, pp. 303–339.
13. Goswami, C. and Talukdar, N. C., *In-situ* mineralization of ahu rice stubble and its effect on succeeding rice and soil properties. In *Biotechnology in Sustainable and Organic Farming*, Shree Publishers, 2004, pp. 313–317.
14. Chanabun, R., Sutcharit, C., Tongkerd, P. and Panha, S., The semi-aquatic freshwater earthworms of the genus *Glyphidrilus* Horst, 1889 from Thailand (Oligochaeta, Almidae) with redescriptions of several species. *ZooKeys*, 2013, **265**, I-76; doi:10.3897/zookeys. 2653911, p. 1–74.
15. Vijayamohanan, Occurrence and density of earthworm in relation to soil factors and altitudes in Kollan district, Kerela. Final Project Report to University Grants Commission, India, 2014, p. 20; www.bjmgovtcollege.in
16. Thakuria, D. *et al.*, Field preservation and DNA extraction methods for intestinal microbial diversity analysis in earthworms. *J. Microbiol. Meth.*, 2009, **76**(3), 226–233.
17. SubbaRao, N. S., *Soil Microbiology Fourth Edition of Soil Microorganisms and Plant Growth*, Oxford & IBH, New Delhi, 1999, p. 407.
18. Cremer, A., *Antibiotic Sensitivity and Assay Tests*, Butterworth, London, 1980, p. 521.
19. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, **227**(5259), 680–685.
20. Lane, D. J., 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics* (eds Stackebrandt, E. and Goodfellow, M.), John Wiley, Chichester, United Kingdom, 1991, pp. 115–175.
21. Zhang, W., Dunkle, J. A. and Cate, J. H. D., Structures of the ribosome in intermediate states of ratcheting. *Science*, 2009, **325**(5943), 1014–1017.
22. Sambrook, J. and Russell David, W., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, p. 3.
23. Magurran, A. E., Measuring biological diversity. *Afr. J. Aquatic Sci.*, 2004, **29**(2), 285–286.
24. Bray, J. R. and Curtis, J. T., An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monographs*, 1957, **27**(4), 325–349.
25. Clarke, K. R., Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.*, 1993, **18**, 117.
26. Hallatt, L., Viljoen, S. and Reinecke, A., Moisture requirements in the life cycle of Perionyx excavatus (Oligochaeta). *Soil Biol. Biochem.*, 1992, **24**(12), 1333–1340.
27. Goullet, P. and Picard, B., The electrophoretic polymorphism of bacterial esterases. *FEMS Microbiol. Rev.*, 1995, **16**(1), 7–31.
28. Kudo, H., Cheng, K.-J. and Costerton, J., Electron microscopic study of the methylcellulose-mediated detachment of cellulolytic rumen bacteria from cellulose fibers. *Can. J. Microbiol.*, 1987, **33**(3), 267–272.
29. Mandels, M. and Weber, J, Physical structure of cellulose. *Adv. Chem. Ser.*, 1969, **96**, 391.
30. Garg, S. and Neelakantan, S., Studies on the properties of cellulase enzyme from *Aspergillus terreus* GN1. *Biotechnol. Bioeng.*, 1982, **24**(3), 737–742.
31. Mandels, M. *et al*., Enzymatic hydrolysis of cellulose: evaluation of cellulase culture filtrates under use conditions. *Biotechnol. Bioeng.*, 1981, **23**(9), 2009–2026.
32. Valle-Molinares, R., Borges, S. and Rios-Velazquez, C., Characterization of possible symbionts in *Onychochaeta borincana* (Annelida: Glossoscolecidae). *Eur. J. Soil Biol.*, 2007, **43**, S14–S18.
33. Knapp, B. *et al*., Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. *Soil Biol. Biochem.*, 2009, **41**(11), 2299–2307.

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